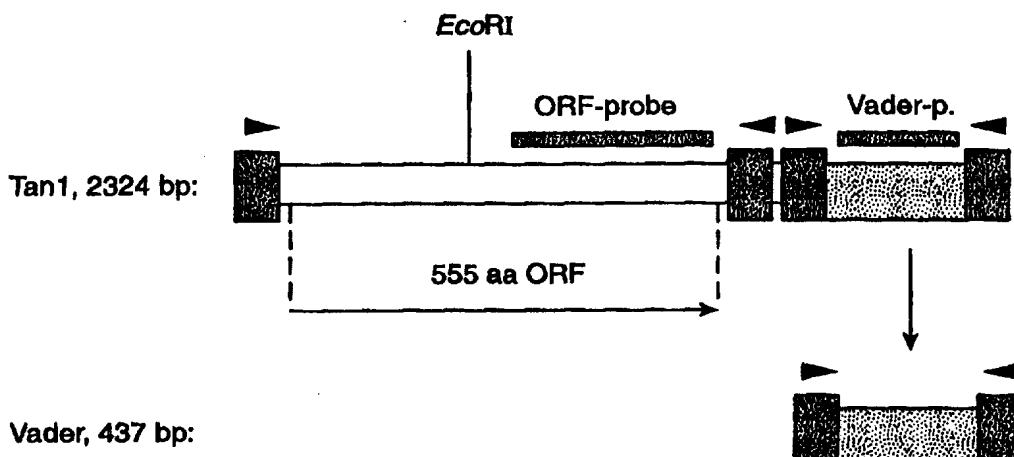




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/81, 9/22, C12Q 1/68, C12N 15/11</b>		A1	(11) International Publication Number: <b>WO 98/08960</b> (43) International Publication Date: <b>5 March 1998 (05.03.98)</b>
(21) International Application Number: <b>PCT/US97/14978</b>		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: <b>25 August 1997 (25.08.97)</b>		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 08/703,077 26 August 1996 (26.08.96) US			
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(54) Title: IDENTIFICATION OF AND CLONING A MOBILE TRANSPOSON FROM *ASPERGILLUS*

## (57) Abstract

There are provided transposable elements isolated from *Aspergillus*. Also provided are fragments comprising the inverted repeat(s) of the transposable elements, such fragments being useful as probes to isolate transposable elements from other filamentous fungi.

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## IDENTIFICATION OF AND CLONING A MOBILE TRANSPOSON FROM *ASPERGILLUS*

### Field of the Invention

10        The present invention is directed at the identification, cloning and sequencing of mobile transposons or transposable elements from *Aspergillus niger* var. *awamori*. The transposable elements, referred to as Vader and Tan1, are approximately 437 base pair (bp) and 2.3 kb elements, respectively. The Vader and Tan1 elements are bounded by inverted repeat sequences of 44 and 45 base pairs, respectively. The transposable 15      elements target a "TA" sequence in target DNA during insertion. In addition, the present invention is directed at the identification, cloning and sequencing of one or more transposable element(s) from other filamentous fungi using as a probe DNA comprising the Vader element 44 bp or the Tan1 element 45 bp inverted repeat isolated from *Aspergillus niger* var. *awamori*. Also provided are methods for utilizing either the Vader or Tan1 20      elements to inactivate genes (for example, by inserting the transposon into the gene to be inactivated), to overexpress a gene (by, for example, inserting a known promoter or other regulatory gene within the inverted repeats of Vader or Tan 1 and allowing the DNA of the IR-promoter-IR to jump in front of (and overexpress) a gene of interest) or to act as an activation marker to, for example, identify new promoters.

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### Background of the Invention

It is well known that transposons are a class of DNA sequences that can move from an episome to a chromosomal site or from one chromosomal site to another. Transposons are known in both prokaryotes, such as bacteria, as well as in eukaryotes, although there 30      have been few transposons isolated from filamentous fungi.

Several groups have looked for transposons in filamentous fungi. The element *pogo*, which exists in multiple copies and at different sites in different strains of *Neurospora crassa*, was described by Schectman (1) and is believed to be a transposon. To date the most characterized transposon in filamentous fungi is Tad. Tad was isolated as a 35      spontaneous mutant in the *am* (glutamate dehydrogenase) gene in an *Adiopodoume* strain of *N. crassa* isolated from the Ivory Coast. To detect mutations caused by insertion of a transposable element, Kinsey and Helber (2) isolated genomic DNA from 33 *am* mutant

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transposable element, Kinsey and Helber (2) isolated genomic DNA from 33 *am* mutant strains which were then screened by Southern analysis for restriction fragment size alterations. In two of the mutant strains, the mutation was shown to be caused by the insertion of a 7 kb element (Tad) into the *am* gene. Subsequently Kinsey (3) demonstrated  
5 that Tad was able to transpose between nuclei of heterokaryons, confirming that Tad was a retrotransposon and that there was a cytoplasmic phase involved in the retrotransposition events. More recently, Cambareri et al. (4) demonstrated that Tad was a LINE-like DNA element with two major open reading frames (ORFs) on the plus strand. Typical of LINE-like elements, Tad had no terminal repeats. Attempts to isolate mobile transposons in  
10 laboratory strains of *N. crassa* were unsuccessful.

A second retrotransposon was cloned by McHale et al. (5), who reported the isolation of CfT-1, an LTR-retrotransposon from *Cladosporium fulvum*. This transposon was 6968 bp in length and bounded by identical long terminal repeats of 427 bp, a 5 bp target site duplication. Virus-like particles were detected which co-sediment with reverse  
15 transcriptase activity in homogenates of this fungus.

Daboussi et al. (6) were the first to successfully use the *niaD* (nitrate reductase) gene as a transposon trap. The *niaD* mutants can be isolated by a direct selection for chlorate resistance (7). The strategy employed was to isolate *niaD* mutants amongst six isolates belonging to different races of the fungus *Fusarium oxysporum*. More than 100  
20 *niaD* mutants were isolated from each isolate and examined for instability. One strain, F24, yielded up to 10% unstable *niaD* mutants. Assuming that the genetic instability of the *niaD* mutants was caused by transposable elements, it seemed plausible that this isolate contained mobile transposons. A stable *niaD* mutant in the F24 was transformed with the cloned *niaD* gene from *A. nidulans* because the *F. oxysporum niaD* gene had not been  
25 cloned. Unstable *niaD* mutants were isolated in transformants containing the *A. nidulans niaD* gene. Two unstable *niaD* mutants were shown by Southern blot analysis to contain a insertion of 1.9 kb in size. Analysis of this element, *Fot1*, revealed it was 1928 bp long, had a 44 bp inverted terminal repeats, contained a large open reading frame, and was flanked by a 2 bp (TA) target site duplication. Very recently, Daboussi et al. (8) have reported the  
30 cloning of a new transposable element from an unstable *niaD* mutant. This element, *FML* (*Fusarium mariner-like*), is 1280 bp long and has inverted repeats of 27 bp. The *FML* element inserts into a TA site and excises imprecisely.

Using the characterization of unstable *niaD* mutants strategy, Lebrun et al. (9) were able to isolate a transposon from *Magnaporthe grisea*. However, in this case the *A.*

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*nidulans niaD* gene which was transformed into *M. grisea* by transformation was used as a transposon trap. The element inserted into the *niaD* gene was shown to belong to a family of *M. grisea* LTR-retrotransposons, Fos1 (Schull and Hamer, unpublished) and Mag1 (Farman and Leong, unpublished). The cloned retro-element was 5.6 kb and the target site 5 (ATATT) was shown to be duplicated. All revertants from this mutant examined had one copy of the LTR left at the point of insertion. A second transposon, Pot2, from *M. grisea* was recently cloned by Kachroo et al. (10). The strategy used to clone Pot2 was to analyze the fingerprint patterns of repetitive DNA's which were cloned from the *M. grisea* genome. A repetitive family present in both rice and non-rice pathogens of *M. grisea* in high copy 10 number was cloned. The element, 1857 bp in size, has a 43 bp perfect terminal inverted repeats (TIR) and 16 bp direct repeats within the TIRs. An open reading frame was shown to display extensive identity to that of Fot1 of *F. oxysporum*. As with Fot1, the Pot2 element duplicates the dinucleotide TA at the target insertion site. Pot2 was shown to be present at a copy number of approximately 100 per haploid genome.

15 Several groups have reported looking without success for transposons in laboratory strains of *A. nidulans* (Kinghorn personnel communication, 5). One explanation for the lack of transposons in laboratory strains is that the desirable features of strain stability required for genetic analysis may preclude strains with mobile transposon. By using the *niaD* gene as a transposon trap we have identified and isolated a transposable element 20 from the industrially important fungus *A. niger* var. *awamori*. This element, Vader, is present in approximately 15 copies in *A. niger* and *A. niger* var. *awamori*. Southern analysis of *A. nidulans* with this element indicates that this transposable element was absent from one laboratory strain and only present as a single copy in a second laboratory strain. These results support the notion that laboratory strains of *A. nidulans* contain very 25 few transposons.

#### Brief Description of the Invention

In accordance with the present invention, novel eukaryotic transposable elements from *Aspergillus niger* var. *awamori* are provided. The larger transposable element, 30 referred to herein as Tan1, is 2.3 kb in size. The smaller transposable element, referred to herein as Vader, is a 437 bp element (SEQ ID NO:3). Vader is found within the larger element Tan1. The Vader transposable element is a 437 bp element which comprises a 44 bp inverted repeat sequence at either end of the transposable element. Tan1 is approximately a 2325 bp element which comprises 45 bp inverted repeats at either 35 terminus and internal IRSs. Tan1 comprises a 555 aa open reading frame (ORF) which

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codes for a transposase which allows the elements (Tan1 or Vader) to "hop" or insert themselves in the genome of a host. The target for insertion of these novel transposable elements is a "TA" sequence in the target DNA for insertion. The "TA" sequence is repeated at either end of the transposon upon insertion of the transposable element into 5 the target DNA. Therefore, the present invention provides the larger Tan1 transposable element as well as the smaller element (Vader) internal thereto, as well as the DNA encoding each.

Another embodiment of the present invention comprises a fragment of the Vader or Tan1 transposable elements which comprise the 44 or 45 bp (respectively) inverted repeat 10 sequences found at either terminus of the transposable element from *A. niger* var. *awamori*, as well as the use of said fragments as probes to hybridize under low stringency conditions to DNA of other filamentous fungi for the isolating and/or cloning of transposable elements from such other filamentous fungi. While the exact 44 bp IR of Vader or the 45 bp IR of Tan1 can be utilized, it is well understood by those skilled in the art that variation of 15 such DNA would also work as a suitable probe. For example, at a minimum, the imperfect direct repeats within the IRs of Tan1 would be suitable to use as probes for isolating transposable elements from other filamentous fungi. Initially the inverted repeat of Vader was used to clone Tan1 using PCR techniques. This work was followed by obtaining a genomic copy of Tan1 from a partial library.

20 Another embodiment of the present invention is the transposase activity coded for by the ORF of Tan1. This transposase is 555 aa (SEQ ID NOS:7 or 14, PCR and genomic, respectively).

In a process embodiment of the present invention there are provided methods for 25 gene tagging comprising using the transposable elements of the present invention (Vader or Tan1 or any transposable element isolated using the IRs of either) to inactivate genes via insertion of the element into a given gene, thus disrupting or inactivating gene expression. Alternatively, the transposable element can be used in activation tagging (to activate or turn on genes) rather than for gene disruption. For example, by inserting DNA coding a promoter into the transposable element and then allowing such transposable 30 element to become inserted 5' to a desired gene, the promoter may be activated to drive the expression of the desired gene product or to turn on cryptic pathways. Additionally, gene tagging can be utilized to activate marker genes by inserting a marker gene within the IRs of a transposon of the present invention. This marker gene can then "hop" into targeted DNA and, if expression of the marker is selected for, it will be possible to identify

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the promoter driving such expression. This may lead to identification of isolation of new strong promoters.

#### Brief Description of the Drawings

5 Fig. 1 shows the Southern blot analysis of unstable *niaD* mutants. PCR-amplified genomic *niaD* gene from four *niaD* mutants and UVK143f were digested with *Bgl*II (sites are 3' of all inserts). Blot probed with 500 bp fragment of *Sall* digested PCR product of *niaD*1 and *niaD*2. Wild-type band hybridizes at 2.5 kb while gene with insertion hybridizes at 2.9 kb. Lanes: 1=MW marker III (Boehringer Mannheim); 2=UVK143f; 3=*niaD*410; 4=*niaD*436; 10 5=*niaD* 587; 6=*niaD*392.

15 Fig. 2 depicts the mapping of Vader insertions within the *niaD* gene. The positions of Vader insertions 1-4 (*niaD*410, *niaD*436, *niaD*587 and *niaD*392, respectively) are shown relative to the six introns of the structural gene coding region. Because the exact site of insertion for Vader-1 and Vader-4 is still unknown, they have been presented using the approximate area of insertion. Relevant restriction sites are shown using the following letters: E=*EcoRI*, S=*Sall*, Sp=*SphI*, K=*KpnI*, and B=*Bgl*II.

20 Fig. 3 shows Southern blot analysis to determine Vader genomic copy number. Four *A. niger* var. *awamori* *niaD* mutants and UVK143f were digested with *EcoRV* to completion. *EcoRV* cuts the Vader sequence once. Hybridization indicates that Vader is present in the genome in more than 14 copies. The hybridizing bands of *niaD* 392, which are different from the other mutants and UVK143f, suggest that the Vader sequence is mobile. Lanes: 1=MW marker III, 2=UVK143f, 3=*niaD*410, 4=*niaD* 436, 5=*niaD* 587, 6=*niaD* 392.

25 Fig. 4. Southern blot to determine presence of Vader sequence in other fungi. Other filamentous fungi, an industrial production strain and *niaD* mutant 392 were digested with *EcoRV* to completion. Low stringency hybridization (32) indicates that sequences homologous to Vader are present in *A. nidulans* (FGSC A237), *A. cinnamomeus*, *A. phoenicis*, *A. foetidus*, an industrial *A. niger* strain. Lanes: 1=MW marker, 2=*A. foetidus*, 3=an industrial glucoamylase production strain of *A. niger* (ETC #2663), 4=*A. niger* var. 30 *awamori* *niaD* mutant 392, 5=*A. phoenicis* (ATCC #11362), 6=*A. nidulans* (FGSC A691), 7=*A. wentii* (ATCC #10593), 8=*A. versicolor*, 9= *A. cinnamomeus* (ATCC #1027), 10=*A. nidulans* (FGSC A237)

Fig. 5. Southern blot to determine Tan1 (transposon from *A. niger*) genomic copy number. Four *niaD* mutants *A. niger* var. *awamori* mutants and UVK143f were digested

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with *EcoRI* to completion. *EcoRI* cuts the Tan1 sequence once. A probe corresponding to the ORF region (see Fig. 9) was used in the hybridization. Hybridization indicates that Tan1 is present as a single copy in the genome. Lanes: 1=MW marker III, 2=UVK143f, 3=*niaD*410, 4=*niaD* 436, 5=*niaD* 587, 6=*niaD* 392.

5 Figs. 6A-6C. Southern blots to determine if the inverted repeats of transposable elements Fot1 and Pot2 will hybridize to elements in *A. niger* var. *awamori*. Four *niaD* mutants *A. niger* var. *awamori* mutants were digested with *EcoRI* to completion. *EcoRI* cuts the Tan1 sequence once. Inverted repeat oligonucleotide probes of Vader (SEQ ID NO:5), Fot1 and Pot2 were labeled with digoxigenin (Boehringer Mannheim). Lanes: 1=MW 10 marker III, 2=*niaD*436, 3=*niaD*587. Blot A (lanes 1-3) and B and C were probed with the labeled inverted repeat probes of Vader, Fot1 and Pot2, respectively.

15 Fig. 7 shows the sequence of the Vader insertion (SEQ ID NO:3) as generated by PCR. Vader was found to be 437 bp in length. The 44 bp inverted repeat of the Vader insert corresponding to SEQ ID NO:4 (the 5' IR) and SEQ ID NO:5 (the 3' IR), respectively, from the 5' end to the 3' end of Vader are underlined, the single mismatch which occurs in the inverted repeats is identified in bold, and the TA 2 bp duplication is shown in bold print. *niaD* sequences flanking the element are shown in lower case letters.

20 Figs. 8A and 8D show the entire DNA sequence of the Tan1 element (SEQ ID NO:6) as generated by PCR, as well as the putative amino acid sequence of the transposase coded for by Tan1 (SEQ ID NO:7). Tan1 as generated by PCR is 2320 bp in 25 length (excluding the unknown nucleotides shown as "N" in the figure) and has a large open reading frame of 1668 bp which encodes for 555 amino acids (SEQ ID NO:7). Tan1 comprises the sequences of four inverted repeats (underlined) similar to those found in Vader.

Fig. 9 shows a schematic presentation of Vader and Tan1 elements. Dark boxes represent the 45 bp (Tan1) and 44 bp (Vader) inverted repeats. The unique *EcoRI* site in the Tan1 element was used for digestion of genomic DNA in Southern analysis (Figs. 5 and 10). Bold, horizontal lines above the Tan1 element indicate the probes corresponding to the end of the ORF and Vader used in Southern analysis shown in Fig. 10 and Fig. 5.

30 Fig. 10 shows Southern analysis of *A. niger* var. *awamori* *niaD* mutants (*niaD*410, *niaD*436, *niaD*587, *niaD*392) and the wild-type UVK143f: lane 1, molecular weight marker III (Boehringer Mannheim); lane 2, UVK143f; lane 3, *niaD*410; lane 4, *niaD*436; lane 5, *niaD*587; lane 6, *niaD*392. This blot was probed for the Vader element (see Fig. 9). When 35 this blot (Fig. 10) was superimposed with the blot shown in Fig. 5, one of the illuminated bands from the Vader-probe hybridization overlaid the single band in the ORF-probe

hybridization indicating that the Tan1 element is composed of contiguous ORF and Vader elements.

Figs. 11A and 11D show the nucleotide sequence (genomic copy) of Tan1 (SEQ ID NO:13). The amino acid sequence encoding the putative transposase (555 aa) (SEQ ID NO:14) is shown below the DNA sequence in the one-letter amino acid code. The inverted repeats are underlined (SEQ ID NOS:1, 2, 15 and 16, respectively, 5' to 3') and the imperfect direct repeats within the inverted repeats are shown with arrows above or below the sequence. The gaps within the arrows indicate the imperfect nucleotides within the direct repeats. Undetermined sequence is denoted in the figure by question marks and in the sequence listing as "N." The figure shows the DNA sequence as 2324 base pairs, excluding the unknown nucleotides indicated by "?" in the figure.

#### Detailed Description of the Invention

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following detailed description of preferred embodiments.

Standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). N connotes any of these nucleotides. As is conventional for convenience in the structural representation of a DNA nucleotide sequence, only one strand is usually shown in which A on one strand connotes T on its complement and G connotes C.

Applicants have isolated two transposable elements from *A. niger* var. *awamori*. The cloned element Vader was identified by screening unstable nitrate reductase (*niaD*) mutants for insertion. This element is present in approximately fifteen copies in the genome of *A. niger* strains examined. In contrast, the Vader element is present in one copy in only one of the two *A. nidulans* strains studied. These results explain why several groups have been unsuccessful in isolating active transposons in laboratory *A. nidulans* strains. A plausible assumption is that "domesticated" strains of *A. nidulans* have lost their transposons due to repeated manipulation of such strains and the possible discarding of aberrant *A. nidulans* strains displaying genetic instability.

The Vader element shows similarities to transposable elements cloned from the plant pathogens Pot1 from *M. grisea* (12) and Fot1 from *F. oxysporum* (8). The target site for duplication in all three fungi is a 2 bp TA sequence. In the case of Fot1, this transposon does not excise precisely. In two *niaD* revertants examined, the excision products retained a 4 bp insertion relative to the wild-type gene (TAATTA versus TA). The insertion studied

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was integrated into an intron, therefore, imprecise excision of Fot1 did not effect the functionality of the *niaD* gene product. There is no published evidence that Pot2 is a functional element.

A homology search made at the nucleotide level gave a strong 60.7% homology 5 between Tan1 and a 1230 bp overlap to the *A. oryzae agdA* gene coding for an  $\alpha$ -glucosidase (33). This homology search revealed that the last 1.2 kb of a total of 5.2 kb of the  $\alpha$ -glucosidase sequence submitted to GenBank is, in fact, part of a novel transposon, hereinafter called *Tao1* (transposon *Aspergillus oryzae*), which also belongs to the *Fot1* family. Only the 5' half of the *Tao1* element is included in the GenBank sequence, thus, for 10 the lack of comparison, the exact size of the inverted repeat cannot be determined. However, it can be concluded that there are 13 bp perfect direct repeats within the inverted repeat. The inverted repeat is flanked by a TA-dinucleotide, suggesting a commonly occurring TA-insertion site. Direct analyses gave only short ORFs, but when the often-occurring stop codons were ignored, a long ORF was obtained which shared over 50% 15 identity to the *Tan1* transposase. Multiple stop codons indicate that the *A. oryzae Tao1* is a defective element. This transposable element from *A. oryzae*, thus, is within the scope of the present invention as, based on the high degree of sequence homology between the *Tan1* and *Tao1*, it is believed that *Tao1* would hybridize to a probe comprising *Tan1* or *Vader* IRs or variations thereof. The sequence of the IR of *Tao1* is provided as SEQ ID 20 NO:17. This IR (*Tao1*) or the IRs from *Tan1* or *Vader* may be used to isolate other transposable elements from filamentous fungi.

In an attempt to determine if there were transposons similar to those reported for *F. oxysporum* and *M. grisea*, synthetic oligomers were made corresponding to the inverted repeats of both *Fot1* (7) and *Pot2* (10). When Southern analysis of *A. niger* var. *awamori* 25 was conducted using the *Vader* 44 bp inverted repeat (SEQ ID NO:5) as a control, no conclusive hybridizations could be detected with either the *Fot1* or *Pot2* oligomeric probe. These results indicate that elements with high identity to *F. oxysporum* *Fot1* and *M. grisea* *Pot2* are not found from *A. niger* var. *awamori* genome.

With regard to the structure of the *Vader* element, elements which transpose 30 directly through DNA copies are typified by having inverted terminal repeats. Elements which transpose through reinsertion of the product of reverse transcription of an RNA copy of the element (retroelements) can be without long terminal repeats such as the *Drosophila* *I* element (for a review see (16)). Alternatively, retrotransposons can have long terminal repeats such as the *Drosophila copia* element. The *Vader* inverted repeats shown in Fig. 7,

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SEQ ID NOS:4 and 5, respectively, have a single mismatch. Elements which transpose through DNA copies typically have open reading frame(s) which encode a transposase activity. The Fot1 element is 1.9 kb in length and the Pot1 element 1.8 kb in length. Both the Fot1 and Pot1 elements have ORF encoding for a putative transposase-like protein.

5 The Vader element, although mobile, does not have an ORF and hence it was deduced that the mobility of Vader was dependent upon a transposase activity present elsewhere in the genome. A synthetic 44 bp oligomer of the inverted repeat of Vader (SEQ ID NO:5) was used to clone, via PCR, a 2.3 kb element. This element, called Tan1 (SEQ ID NO:6), comprises four inverted repeats (SEQ ID NOS:1, 2, 15 and 16 from 5' to 3', respectively) similar to those in Vader and has a unique organization IR-ORF-IR-IR-Vader-IR. Tan1 is 10 2324 bp in length and has a large open reading frame (1668 bp) which encodes a putative transposase comprising 555 amino acids (shown in SEQ ID NOS:7 and 14), which is homologous to Fot1 and Pot2 transposases. Immediately 3' to the second IR (SEQ ID NO:2), which bounds the transposase, is a copy of the Vader element. We hypothesize 15 that at some stage the independent Vader element, although inactive by itself, has arisen from Tan1, resulting in current strains with only one copy of Tan1 providing transposase activity and numerous mobile copies of Vader dispersed in the genome.

Thus, applicants have been the first to identify a transposable element(s) with certain *Aspergillus* species. These transposable elements are believed to be quite useful in 20 the development of gene tagging systems for *Aspergillus* or other microorganisms. Basic requirements for developing a gene tagging system are that the tagging element can be distinguished from the endogenous elements, it displays little sequence specificity for transposition and that excision is followed by integration at a new site. More refined 25 tagging systems include ability to monitor excision and reinsertion by, e.g., activation of antibiotic resistance genes and ability to stabilize the mutations by, e.g., a two transposons system (23, 24 and 25).

For development of a tagging system for *Aspergillus*, it is proposed that the system is tested first in *A. nidulans*, which we have already shown does not have endogenous 30 Tan1 or Vader sequences. However, at this stage the Vader element is altered from the original in such a way that the same construction can be later used in *A. niger* var. *awamori* and be distinguished from the endogenous Vader elements.

In a model tagging system using Vader as the "mutator," a first vector can be constructed for expression of the Vader element, similar to the non-autonomous maize Dc. The internal sequence of the Vader element is altered to contain translation initiation and 35 stop codons in three different frames. This sequence can later be used as a recognition

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site for a probe in PCR analysis of the mutants. This altered Vader element, Vader-S, is inserted within an expression cassette conferring antibiotic resistance such as hygromycin resistance. Since excision of Vader may not always be precise, Vader-S is inserted in the promoter area (e.g., *o/iC*) between the transcription and translation initiation sites. This  
5 disrupted hygromycin phosphotransferase cassette is flanked by marker genes - or alternatively the marker gene upstream of the hygromycin promoter can be placed within Vader. These marker genes can be used for monitoring whether the hygromycin gene, and Vader within it, have integrated in full length. A vector, for example, Vector I, containing these elements will be transferred to *A. nidulans* and transformants expressing the two  
10 marker genes, but sensitive to hygromycin, are selected. Screening of mutants at later stages is easier, if the transformant selected for mutagenesis has only one to two copies of Vector I sequences integrated in its genome.

A transformant with only a few (preferentially one) intact Vader-S/hygromycin phosphotransferase cassettes integrated in its genome is retransformed with Vector II,  
15 which is an autonomously replicating vector carrying the transposase encoding gene. The autonomously replicating vector, pHELP, used as a basis for DNA construction work, can be segregated away by methods known to those skilled in the art. This enables stabilization of the Vader-S element after the mutagenesis step. Vader-S is activated by a transposase (from Tan1) in pHELP, which can be monitored by activation of the  
20 hygromycin resistance gene. Tan1 is not cloned into the vector in full length to disrupt its mobility. Again, Vector II contains a marker gene used for screening of transformants and also for monitoring its segregation after the sporulation phase.

Marker genes can either complement host mutations or be dominant markers such as benomyl<sup>R</sup>, acetamidase or β-glucuronidase (GUS).

In a model system for gene tagging the target gene for mutagenesis should be one with a simple plate screen, e.g., disruption of the *niaD* gene (by insertion of Vader), which can be screened by selection of chlorate resistant mutants and the gene disruption can be further mapped by a plate test using different nitrogen sources (no growth on nitrate, growth on nitrite, xanthine and uric acid). Another target gene for mutagenesis could be an  
30 acid protease gene. It has been shown previously for *A. niger* that disruption of this one protease is sufficient to abolish halo formation almost completely on skim milk plates.

The advantage of using transposon tagging is that the mutants produced can be identified by subsequent isolation of the mutated gene. There are several methods available for PCR amplification of genomic sequences when only one end of the sequence  
35 is known - which, in this case, is the transposable element. PCR methods developed for

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genomic walking are, e.g., "Inverse PCR" (27 and 28), "Vectorette PCR" (29) and "Panhandle PCR" (30).

Setting up the transposon tagging system can be followed by studies of excision frequency, environmental influences on transposition frequency (24, 31), activation of the 5 transposase by a heterologous promoter and effect of altered inverted repeats on transposition.

Transposon tagging does need to be applied for inactivation of genes.

Alternatively, tagging can be used to insert promoter sequences in Vader and therein activate genes. A third option is to insert a promoterless marker gene in Vader, in which case the transposon can be used in search for novel, strong fungal promoters.

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## Experimental

### Materials and Methods

**Strains.** Vader and Tan1 elements were isolated from *Aspergillus niger* var. awamori UVK143f, derived from Northern Regional Research Laboratories (NRRL) #3112. 15 *E. coli* JM101 [F' *traD36 lac1*<sup>q</sup> Δ(*lacZ*)*M15 proA*<sup>+</sup>*B*<sup>+</sup> /*supE thi* Δ(*lac-proAB*)] and *Epicurian coli* SURE 2 (Stratagene Cloning Systems, La Jolla, CA) were used for propagation of Vader and Tan1 subclones, respectively.

Spontaneous chlorate resistant mutants were derived from *Aspergillus niger* var. awamori UVK143f (NRRL #3112). The following *Aspergillus* strains were obtained from the 20 ATCC: *A. cinnamomeus* (ATCC #1027), *A. wentii* (ATCC #10593), and *A. phoenicis* (ATCC #11362). *A. nidulans* (FGSC #A237), a nitrate reductase structural gene mutant (*niaD15*), and *A. nidulans* (FGSC #A691), a tryptophan requiring mutant (*trpC801*), were obtained from Fungal Genetics Stock Center (FGSC), Dept. of Microbiology, University of Kansas Medical Center. *A. versicolor*, *A. foetidus*, and a proprietary *A. niger* glucoamylase strain are from the Genencor International Inc. culture collection.

**Mutant Selection.** Spore suspensions ( $1 \times 10^8$ ) of UVK143f were plated on CM agar (11) containing 600 mM  $\text{KClO}_3$  and 10 mM glutamic acid. Chlorate ( $\text{KClO}_3$ ), a toxic analog of nitrate, allows selection of mutants in the nitrate assimilation pathway by chlorate resistance. Plates were incubated at 37°C until individual colonies of spontaneous mutants could be identified. Single mutants resistant to  $\text{KClO}_3$  were allowed to sporulate on CM 30 plates and spores from these plates were then streaked onto minimal media (11) with various sole nitrogen sources (10 mM):  $\text{NaNO}_3$  (nitrate),  $\text{NaNO}_2$  (nitrite), hypoxanthine, uric acid or  $\text{NH}_4\text{Cl}$  (ammonium chloride). Each of these compounds are intermediate products

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of the nitrate assimilation pathway. *niaD* mutants were identified as those resistant to KClO<sub>3</sub> and able to grow in the presence of all pathway intermediates, except for NaNO<sub>3</sub>.

**Isolation of Vader via PCR Amplification.** Genomic DNA of *A. niger* var. *awamori* *niaD* mutants and UVK143f was used as template (see Southern Analysis). Primers (50 pmol) used for amplification of the *niaD* gene were NiaD1 (position 142-165 relative to the initiation site of *niaD*): 5'-CCAACCGAGTCCTCAGTATAGAC-3' (SEQ ID NO:8) and NiaD2 (position 2738-2715): 5'-CAACGCTTCATAGGCGTCCAGATC-3' (SEQ ID NO:9). Deep Vent (exo-) DNA polymerase (New England Biolabs) was used with the buffer and dNTPs provided by the manufacturer. For optimal amplification of the *niaD* gene the reaction mixture contained 4 mM MgSO<sub>4</sub>. Denaturation of template DNA, 2 min. at 94°C, was followed by 30 cycles of denaturation (30 sec. at 94°C), annealing of primers (45 sec. at 55°C) and extension (4 min. at 72°C). PCR fragments were purified from gel using the Qiaex DNA gel extraction kit (Qiagen), digested and used for restriction enzyme analysis by standard procedures (12).

**Confirmation of Excision Foot Print by PCR Amplification and Sequencing.**

Template DNA from *niaD*436 was used in a PCR reaction in an attempt to amplify both the larger *niaD* sequence with an insert and the shorter *niaD* fragment resulting from excision of the Vader element. The PCR reaction was conducted as previously described, except for using primers MA003 (positions 359-378): 5'- ATATGAATTCCCTTCTTGACTTCCCCGGAAC-3' (SEQ ID NO:11) and NiaD5 (position 1125-1144): 5'-ATATAAGCTTGTCACTGGACGACATTTCAG-3' (SEQ ID NO:12). The gel purified fragment (ca. 800 bp) resulting from the excision event was submitted for sequencing.

**Isolation of Tan1 via PCR Amplification.** Fungal genomic DNA for PCR and Southern analyses was isolated from mycelia grown in CSL supplemented with 5% fructose (21). Genomic DNA of *A. niger* var. *awamori* *niaD* 436 mutant (22) was used as a template. A single primer (100 pmol), IR1, was used for amplification of Tan1. The 54-mer IR1 was derived from the 44 bp inverted repeat sequence of Vader preceded by a restriction enzyme recognition site for EcoRI: 5'-ATATGAATT CACGTAAATCAA CGGTCGGACG GGCCACACGG TCAGGGGGGC CATC-3' (SEQ ID NO:10). Deep Vent (exo-) DNA polymerase (New England Biolabs) was used with the buffer and dNTPs provided by the manufacturer. Denaturation of template DNA, 10 min. at 94°C, was followed by 30 cycles of denaturation (1 min. at 94°C), annealing of primers (1 min. at 55°C) and extension (6 min. at 72°C). PCR fragments were purified from agarose gels

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using the Qiaex DNA gel extraction kit (Qiagen) and subcloned as blunt-ended inserts into EcoRV cut pSL1180 (Pharmacia Biotech).

**Estimation of *niaD* Mutant Reversion Frequency.** Spores from *niaD* mutants *niaD*392, *niaD*410, *niaD*436 and *niaD*587 were streaked onto minimal media containing NaNO<sub>3</sub> as a sole nitrogen source. Nitrate non-utilizing colonies of *niaD* mutants, which had a spidery appearance and did not sporulate, were streaked onto CM containing 600 mM potassium chlorate (KClO<sub>3</sub>) and incubated to confluence at 37°C. Ten-fold dilution series of spore suspensions (in 0.8% NaCl-0.25% Tween 80) of *niaD*392, *niaD*410, *niaD*436, *niaD*587 and UVK143f wild-type spores were plated on minimal media with nitrate (10 mM) to determine reversion frequency, and on CM to determine viability.

**Southern Analysis.** Genomic DNA for PCR and Southern analysis was isolated (13) from mycelia grown in CSL (13), which contained 600 mM KClO<sub>3</sub> in order to reduce reversion of *niaD* back to the wild-type during cultivation. DNA (10 µg) was digested with either BglII, which leaves the insertion intact in the *niaD* gene, or with EcoRV, which cuts the insertion element (Vader) once, and thus enables determination of its copy number in the genome. Genomic DNA (approximately 10 µg) of *A. nidulans*, *A. cinnamomeus*, *A. versicolor*, *A. wentii*, *A. phoenicis*, *A. foetidus* and of an industrial *A. niger* strain were digested with EcoRV to obtain an estimate of Vader copy number in these fungal genomes. The digested and gel-separated DNA was transferred to a positively-charged nylon membrane (Boehringer Mannheim) by capillary action.

The DNA probe for the *niaD* gene was derived from the PCR product (UVK143f DNA template amplified with primers NiaD1 (SEQ ID NO:8) and NiaD2 (SEQ ID NO:9)), which was digested with Sall, resulting in a 528 bp probe fragment. The probe for the insertion element, Vader, was derived from a PCR reaction in which *niaD*436 DNA was used as a template. This PCR product was purified and digested with Sall and SphI and subcloned into the vector pUC19. This subclone was digested with Scal and XbaI to yield a 236 bp fragment which was used for estimation of the copy number of Vader sequences in the genomes of various fungi.

A DNA labeling and detection kit (Genius1, Boehringer Mannheim) was used for random primed labeling of probe DNA with digoxigenin, and for detection with alkaline-phosphatase labeled antibody to digoxigenin.

Hybridization and washing conditions for homologous probes were conducted as recommended by the manufacturer using hybridization buffer without formamide at 68°C (Boehringer Mannheim). Hybridizations for heterologous Southern analysis (i.e., analysis

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of DNA from other *Aspergillus* sp.) was conducted using hybridization buffer with 25% formamide at 37°C. Washes were performed as in stringent wash protocol.

**Nitrate Reductase Assays.** Nitrate reductase assays were performed as described in Dunn-Coleman, et al. (18).

5       **DNA Analysis and Sequence Determination.** Sequences were determined using fluorescent-labeled dideoxynucleotide terminators and *Taq* cycle sequencing on the 373A sequencer (ABI). Commercially available universal and reverse (New England Biolabs) primers were used. Alignment of sequences and prediction of amino acid sequences were performed using DNASTAR (DNASTAR, Inc.). The nucleotide and deduced amino acid 10 sequences were analyzed and compared to those in GenBank, EMBL and Prot-Swiss using Fast A and BLAST programs (Genetics Computer Group, Inc. software package, Madison, WI).

15       **Other Probes Used for Southern Analysis.** The Tan1 probe was prepared by digesting Tan1 with *Hind*III and *Stu*I resulting in a 650 bp fragment corresponding to the 3' end of the transposase coding region (ORF-probe in Fig. 9). The Vader element was digested with *Xba*I and *Scal* to yield a 236 bp fragment to be used for recognition of internal Vader sequence in Southern analysis (Vader-probe in Fig. 9).

20       **Southern Analysis to Determine Tan1 Copy Number.** *Aspergillus* genomic DNA (10 µg) was digested with *Eco*RI, which cuts the Tan1 element once in the transposase coding region and upstream of sequences corresponding to the Vader and Tan1 probes used in hybridizations (Figs. 5, 9 and 10). DNA labeling and detection kit (Genius 1, Boehringer Mannheim) was used for random primed labeling of probe DNA with digoxigenin and for detection with alkaline-phosphatase labeled antibody to digoxigenin. Hybridization and washing conditions were conducted as recommended by the 25 manufacturer (Boehringer Mannheim).

30       **Isolation of Tan1 from a Partial Genomic Library.** It was known from the sequence of the PCR-amplified Tan1 element that Tan1 did not have restriction enzyme recognition sites for *Bgl*II and *Xho*I. A *Bgl*II-*Xho*I digested Southern blot of *Aspergillus niger* var. *awamori* genomic DNA, hybridized with the 650 bp *Hind*III-*Stu*I Tan1 probe, resulted in identification of a 4.5 kb genomic fragment containing Tan1. *A. niger* var. *awamori* *niaD436* DNA was digested with *Bgl*II and *Xho*I and fragments in a size range of 4-5 kb were cloned into pSP73 (Promega). This partial genomic library was screened by colony hybridization using the nonradioactive nucleic acid labeling and detection system from Boehringer Mannheim.

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**Example 1****Isolation of Spontaneous High Frequency Reverting  
*niaD* Mutants of *A. niger* var. *awamori***

Assuming that *niaD* mutants which arise from the insertion of a transposable element would be unstable, a total of 152 *niaD* mutants, isolated on the basis of spontaneous resistance to chlorate were characterized. To determine if the *niaD* mutation was unstable, spores from 43 *niaD* mutants were plated onto medium with nitrate as the sole nitrogen source. Fourteen of the mutants reverted to the wild-type phenotype at a frequency of greater than  $1 \times 10^{-5}$ . Table 1 summarizes the *niaD* mutant reversion studies.

10 **Table 1**

<u>Mutant</u>	<u>Conidia Plated No. x 10<sup>3</sup></u>	<u>No. Wild-Type Colonies</u>	<u>Reversion Frequency x 10<sup>-4</sup></u>
<i>niaD</i> 392	2.9	27	93
<i>niaD</i> 410	7.7	5	6.5
<i>niaD</i> 436	3.7	164	443
<i>niaD</i> 587	18.9	12	6.3

There appeared to be two classes of *niaD* mutants which reverted at high frequency. The *niaD* mutants *niaD*436 and *niaD*392 reverted at high frequency, while mutants *niaD*410 and *niaD*587 yielded smaller numbers of revertant colonies.

The level of nitrate reductase activity was determined using the assay described in (18) from revertant colonies isolated from the *niaD* 436 mutant. Nitrate reductase activity was detected in 14 of 15 revertants analyzed (see Table 2). A spectrum of activities was detected, suggesting that excision of Vader may not always be precise.

25 **Table 2**

<u>Strain</u>	<u>% Nitrate Reductase Activity Compared to Wild-Type</u>
UVK143f (wild-type)	100
<i>niaD</i> 436 ( <i>niaD</i> mutant)	ND <sup>1</sup>
Revertants of <i>niaD</i> 436:	
1	34.7
2	42.8
3	27.7
4	3.5
5	ND <sup>1</sup>

<sup>1</sup> Activity non-detectable

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Strain	% Nitrate Reductase Activity Compared to Wild-Type
6	47.4
7	90.4
8	9.8
9	25.4
10	28.9
11	38.2
12	6.9
13	71.7
14	71.7
15	49.7

**Example 2**Cloning of a Vader Element

To determine if an insertion sequence was located within the *niaD* gene, two primers were synthesized. The first primer, niaD1 (SEQ ID NO:8), corresponded to position 142-165 of the *niaD* gene, and niaD2 (SEQ ID NO:9) corresponded to position 2738-2715 of the *niaD* gene. Genomic DNA was isolated from 14 unstable *niaD* mutants. This genomic DNA served as a template for the PCR primers. PCR reaction products with 4 *niaD* mutants (410, 436, 587 and 392) revealed an approximately 440 bp insertion (Vader) in the *niaD* gene.

For Southern blot analysis, genomic DNA isolated from the wild-type and four *niaD* mutants (410, 436, 587 and 392) was digested with BgIII. The probe used was a Sall digestion fragment of the 500 bp PCR product generated using the niaD1 (SEQ ID NO:8) and niaD2 (SEQ ID NO:9) oligomeric probes. The probe hybridized to a 2.5 kb fragment with wild-type DNA (lane 5, Fig. 1). In the case of the *niaD* mutants 410 (lane 1, Fig. 1), 436(lane 3, Fig. 1) and 392 (lane 4, Fig. 1), the probe hybridized to a 2.9 kb fragment. These results indicate that these three *niaD* mutants contain an approximately 440 bp insertion. Interestingly, with the mutant *niaD*587, the probe hybridized to both a 2.5 kb and 2.9 kb fragment, although mycelium had been grown in the experiment in the presence of KClO<sub>3</sub> to favor growth of the *niaD* mutant and not revertant cells, the detection of two hybridizable sequences indicated that in some cells Vader had been excised from the *niaD* gene.

The approximate location of the insertion was determined in each of the four unstable *niaD* mutants by restriction mapping analysis. The location of the insertion in each of the four mutants examined is shown in Fig. 2. All four mutants had an approximately 440 bp insertion located at different sites within the *niaD* gene.

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### Example 3

#### Determination of Vader Copy Number

To determine the Vader copy number a 236 bp Scal-XbaI internal fragment of Vader-2 (cloned from the mutant *niaD*436) was hybridized to EcoRV cleaved genomic DNA. There is only one EcoRV site within the Vader transposon. Southern blot analysis indicated that there are approximately fifteen copies of Vader sequences in the genome of *A. niger* var. *awamori*. (Fig. 4). The Vader sequences were integrated at identical genomic locations in the three *niaD* mutants, 410, 436 and 587. However, in the *niaD*392 mutant, Vader sequences were located in five different locations compared to the three *niaD* mutants examined. This result was somewhat surprising considering that all four *niaD* mutants were isolated from the same strain, but provides good evidence for the high mobility of the Vader element in this strain. When a proprietary *A. niger* glucoamylase production strain (ETC #2663) was also examined, approximately 15 hybridization signals could be detected. Although some of the hybridization patterns appeared to be identical, clear differences could be seen between *A. niger* var. *awamori* and *A. niger*.

### Example 4

#### Isolation of Vader in Other Fungal Species

In an attempt to determine if this transposable element was found in other filamentous fungi, genomic Southern blot analysis was performed using the 236 bp fragment (XbaI-Scal) of Vader sequence as per Example 3, as a probe (Fig. 5). Two strains of *A. nidulans* were obtained from Fungal Genetics Stock Center (FGSC), FGSC #A691, a nitrate reductase structural gene mutant (*niaD*15), and FGSC #A237, a tryptophan-requiring mutant (*trpC*801). No hybridization signals could be visualized with strain A691, and a single strong hybridization signal could be detected with strain A237. These results support the notion that the lack of success in cloning transposable elements from laboratory strains of *A. nidulans* is due to low copy number or absence. Similarly, only one hybridization signal could be detected in *A. foetidus* and *A. phoenicis*, while two hybridization signals were detected in *A. cinnamomeus*. No hybridizations could be detected in *A. wentii* and *A. versicolor*. In addition, no hybridization signals could be detected with *Humicola grisea* var. *thermoidea*, *Neurospora crassa* and *Trichoderma reesei* (results not shown). These results indicate that the Vader element is most commonly found in *A. niger* var. *awamori* and *A. niger*.

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### Example 5

#### Excision of the Vader Element

Part of the *niaD* gene from *niaD436* containing the Vader element was amplified using PCR. The PCR amplification resulted in the expected 1200 bp fragment of the Vader element flanked by *niaD* sequences and a shorter 800 bp fragment resulting from the excision event. Sequencing of the shorter fragment indicated that the Vader element had excised precisely. However, when several revertants of *niaD436* and *niaD410* were assayed for their nitrate reductase activity (18), a spectrum of activities was detected, suggesting that excision of the Vader element may not always be precise (results not shown).

### Example 6

#### Isolation of Tan1

The previously isolated Vader element, although mobile, did not have an ORF encoding transposase activity presumed to be required for excision (22). This observation led to a search for a transposase-encoding larger element, thus an oligomer corresponding to the Vader inverted repeat was synthesized and used for PCR amplification of the genomic *A. niger* var. *awamori* DNA. The PCR amplification resulted in the generation of three DNA fragments: the 0.4 kb Vader element, as expected, and fragments of 1.9 kb and 2.3 kb in length.

Both of the larger PCR-generated fragments were sequenced and the sequences were identical with an exception that the 2.3 kb fragment had an additional 400 bp at the 3' end. Surprisingly this additional sequence at the 3' end was a Vader element, which differed only by a few nucleotides from the previously isolated Vader. The 5' end sequence, shared by both of the 1.9 kb and 2.3 kb fragments, had a single ORF (1668 bp) coding for a protein of 555 amino acids flanked by inverted repeats (IRs). Thus, the 1.9 kb fragment, devoid of the Vader element, had an organization of IR-ORF-IR. The larger 2.3 kb fragment had a unique organization, IR-ORF-IR-IR-Vader-IR, with a total of four inverted repeats (Figs. 9 and 11). In this larger element the two central inverted repeats, side by side, potentially form a tight hairpin structure, and despite many sequencing attempts with varying conditions, we were unable to determine the sequence between the two inverted repeats. However, the overall length of the PCR product, as determined by electrophoresis, corresponded to the size of the sequence shown in Fig. 11, suggesting that the two central contiguous IRs are not separated by a large segment of DNA.

Due to the organization of the 1.9 kb and 2.3 kb fragments, it was believed that the 1.9 kb fragment could have arisen in PCR from a partial amplification of the 2.3 kb

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fragment if the 3' IR-primer had annealed to the first central IR instead of the IR in the end of the Vader element. Southern analysis was conducted in order to determine if the 1.9 kb element existed in the genome without the associated Vader element, or whether it was a PCR-artifact derived from a partial amplification of the 2.3 kb element. The two probes used in Southern analysis corresponded to the internal sequence of Vader and to the carboxyterminal part of the ORF (Fig. 9). The genomic DNA from *A. niger* var. *awamori* *niaD* mutants and UVK143f were digested with *Eco*RI, which cuts once in the coding region of the ORF upstream from the ORF-probe and does not cut Vader. The Southern analysis showed numerous bands for the Vader element (Fig. 10), similar to previous Southern analyses (22). However, only one fragment lit up with the probe corresponding to the ORF and a fragment of the same size (1.6 kb) was recognized by the Vader probe (Fig. 10). It was concluded that the actual element in the genome was the 2.3 kb fragment and that the shorter 1.9 kb had only been a PCR-artifact. The isolated 2.3 kb fragment was designated as Tan1.

A genomic clone of the Tan1 element (2.3 kb) was isolated from a partial genomic library. Restriction enzymes, which were shown not to have any recognition sites in the PCR-amplified Tan1, were used separately and in combinations in Southern analysis of the genomic DNA. A double digestion with *Bgl*II and *Xba*I resulted in a relatively short, 4.5 kb, fragment which hybridized with the ORF-specific probe (data not shown). Genomic DNA fragments cleaved by *Bgl*II and *Xba*I and between 4 kb and 5 kb in size were cloned into pSP73 (Promega). The correct clone containing the Tan1 element was isolated by colony hybridization using the ORF-specific probe. Differences between the sequences of the genomic clone and the PCR-generated Tan1 were minor, even for the flanking IRs which were almost identical even though in the PCR-generated Tan1 the IRs were derived from the Vader IRs (PCR primers). It was seen from the genomic clone of Tan1 that immediately outside of the terminal IRs there were TA-dinucleotides, suggesting a TA target site and its duplication upon insertion. Sequence of the Tan1 genomic clone is shown in Figs. 11A and 11B [SEQ ID NO:13 (DNA) and SEQ ID NO:14 (amino acid)].

#### Example 7

##### Insertional Inactivation/Gene Tagging

Vader was cloned by insertional inactivation of the target gene *niaD*, which encodes nitrate reductase. The target sequence for integration of Vader is TA, a sequence which must be very common in the genome of fungi. Nitrate reductase mutants cannot grow on nitrate and in consequence are resistant to the toxic analog of nitrate,  $KClO_3$ .

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It is possible that one of the reasons heterologous protein production in fungi is lower than that of homologously produced protein using the same promoter is that the heterologous protein is being degraded by the cell. If there are genes whose products are responsible for degrading/sequestering foreign protein, it would be advantageous to  
5 inactivate those genes. In order to achieve this, a strain is constructed using gene disruption, which lacks the Tan1 gene. Such strain is then used to transform and express a heterologous protein such as the mammalian chymosin protein. It would be advantageous if the activity of such genes could be visualized or selected for on petri dishes. For example chymosin produced in *A. niger* results in a halo of clearing around a colony grown  
10 on skim milk. (See US Patent 5,364,770, the disclosure of which is incorporated herein by reference.)

Having transformed the strain with a construct comprising the desired heterologous protein or polypeptide, one would transform the strain a second time with Vader and Tan1 appropriately modified for gene tagging purposes.

15 The transformants are then plated on medium which can be used to visualize heterologous protein production, such as skim milk plates in the case of chymosin.

The plates are then screened for increased halo size, which is the result of inactivation of a gene whose product limits foreign protein production.

20 The inactivated gene can be cloned using the transposon sequences as a marker for cloning strategies. (See generally (19).)

#### Example 8

##### Elevation of Gene Expression Using Transposons

A reason that heterologous protein production is lower than expected in fungi is presumed to be that genes essential for foreign (heterologous) gene production are NOT  
25 expressed at sufficiently high levels in the fungi.

In order to overcome this problem, utilizing the transposable element(s) of the present invention, a strain is constructed in which the native Tan1 gene is inactivated by gene disruption.

This strain is used to express a heterologous protein whose expression can be  
30 easily visualized, such as chymosin (US Patent 5,364,770). A second transformation is made with Vader and Tan1, appropriately modified for gene tagging purposes. The internal sequence of Vader is replaced by a promoter sequence. One of the many integration events possible will be the integration of this promoter carrying Vader element into 5' to a gene beneficial to heterologous protein (e.g., chymosin) expression or secretion. Upon  
35 insertion, this beneficial gene is activated and such integrant colonies can be screened for,

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**e.g., increased halo size (chymosin). The activated gene can be cloned using the transposon sequences as a marker for cloning strategies.**

References

1. Schectman, M.G. (1987) *Mol. Cell. Biol.* **7**:3168-3177
2. Kinsey, J.A. & Hebler, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**:1929-1933
3. Kinsey, J.A. (1993) *Proc. Natl. Acad. Sci. USA* **90**:9384-9387
4. Cambareri, E.B., Helber, J. & Kinsey, J.A. (1994) *Mol. Gen. Genet.* **242**:658-665
5. McHale, M.T., Roberts, I.N., Noble, S.M., Beaumont, C., Whitehead, M.P., Seth, D. & Oliver, R.P. (1992) *Mol. Gen. Genet.* **233**:337-347
6. Daboussi, M.J., Langin, T. & Brygloo, Y. (1992) *Mol. Gen. Genet.* **232**:12-16
7. Cove, D.J. (1976) *Heredity* **36**:191-203
8. Daboussi, M.J. & Langin, T. (1994) *Genetica* **93**:49-59
9. Lebrum, M.-H. Chumley, F. & Valent, B. (1994) *Fungal Genetics News Letter* **41A**:52
10. Kachroo, P., Leong, S.A. & Chattoo, B.B. (1994) *Mol. Gen. Genet.* **245**:339-348
11. Rowlands, R.T. & Turner, G. (1973) *Mol. Gen. Genet.* **126**:201-216
12. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 1.11-1.85
13. Timberlake, W.E. & Barnard, E.C. (1981) *Cell* **26**:29-37
14. Yanisch-Peron, C., Vieira, J. & Messing, J. (1985) *Gene* **33**:103-119
15. Meselson, M. & Yuan, R. (1968) *Nature* **217**:1110-1114
16. Charlesworth, B., Snlegowski, P. & Stephan, W. (1994) *Nature* **371**:215-220.
17. Fedoroff, N.V., Furtek, D.B. & Nelson, O.E. (1984) *Proc. Natl Acad. Sci. USA* **81**:3829-3835
18. Dunn-Coleman, N.S., Tomsch, A.D. & Garrett, R.H. (1981) *Molec. Gen. Getet.* **182**:234-239
19. Walden, R. & Schell, J. (1994) *Agro-Food-Industry-Hi-Tech*, Nov/Dec:9-12
20. Gems, D.H., Johnstone, I.L. & Clutterbuck, A.J. (1991) *Gene* **98**:61-67
21. Dunn-Coleman, N.S., Bloebaum P., Berka, R.M., Bodie, E., Robinson, N., Armstrong, G., Ward M., Przetak, M., Carter, G.L., LaCost, R., Wilson, L.J., Kodama, K.H., Baliu, E.F., Bower, B., Lamsa, M. & Heinsohn, H. (1991) "Commercial levels of chymosin production by *Aspergillus*," *Bio/Technology* **9**:976-981
22. Amutan, M., Nyssonnen, E., Stubbs, J., Diaz-Torres, M.R. & Dunn-Coleman, N. (1996) "Identification and cloning of a mobile transposon from *Aspergillus niger* var. *awamori*," *Curr. Genet.* **29**:468-473

-- 23 --

23. Bancroft, I., Bhatt, A., Sjodin, C., Scofield, S., Jones, J. & Dean, C. (1992) *Mol. Gen. Genet.* **233**:449-461
24. Bancroft, I. & Dean, C. (1993) *Mol. Gen. Genet.* **240**:65-67
25. Long, D., Martin, M., Sundberg, E., Swinburne, J., Puangsomlee, P. & Coapland, G. (1993) *Proc. Natl. Acad. Sci.* **90**:10370-10374
26. Berka et al. (1990) *Gene* **86**:153-162
27. Ochman, H., Gerber, A.S. & Hart, D.L. (1988) *Genetics* **120**:621-623
28. Williams, J.F. (1989) *Biotechniques* **7**:762-769
29. Arnold, C. & Hodgson, I.J. (1991) *PCR Methods Appl.* **1**:39-42
30. Jones, D.H. & Winistorfer, S.C. (1993) *PCR Methods Appl.* **2**:197-203
31. Brujin, F.J. & Lupski, J.R. (1984) *Gene* **27**:131-149
32. Brown, T. *Current Protocols in Molecular Biology*, Supplements 21, 24, 26 and 29
33. Minetoki, T., Gomi, K., Kitamoto, K., Kumagai, C., Tamura, G. (1995) "Nucleotide sequence and expression of alpha-glucosidase-encoding gene (*agdA*) from *aspergillus oryzae*," *Biosci. Biotechnol. Biochem.* **59**:1516-1521

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### Sequence Listing

(1) GENERAL INFORMATION:

(i) APPLICANT: Amutan, Maria  
Dunn-Coleman, Nigel  
Nyyssonen, Eini M.

(ii) TITLE OF INVENTION: Identification of and Cloning a Mobile Transposon from Aspergillus

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:

(A) NAME: Genencor International, Inc.  
(B) STREET: 925 Page Mill Road  
(C) CITY: Palo Alto  
(D) STATE: CA  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 94304

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US  
(B) FILING DATE: August 16, 1996  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Horn, Margaret A.  
(B) REGISTRATION NUMBER: 33,401  
(C) REFERENCE/DOCKET NUMBER: GC270-2

(xi) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 846-7536  
(B) TELEFAX: (415) 845-6504

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACGTAATCAA CGGTCGGGCG GGCCACACGG TCAGGCAGGC CACCC

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(2) INFORMATION FOR SEQ ID NO: 2:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATGGCCCGC CTGACCGTGT GGCCCGCCCG ACCGTTGATT ACGT

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 437 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACGTAATCAA CGGTCGAACG GGCCACACGG TCAGGCGGGC CATCCTGAAA TCCCATATAA	60
AAGATGTCTT GGGGATTCTA TTATATATCA ACCAGTACTA CTTCTATGAA GCTCTAACTT	120
TGTAGATAGT TATATATATA AGAATAAGTA TTCCATGAAT TTTTCAGATT TTAGAATTAA	180
TACTTTGATA ATGAAACCAG ATTCTTATAT AAAACATATA AATACAGATA TTGTAATATG	240
ATAAGTCCAT AAGTAAAAGT ATATTCAATT TTAGAAGGTA TATAGATATT ATTTATATTA	300
TTTAAAATCT ATATAGAAGA AATCTAATTC TTCTAGACCT GGATGGTAGA GATATATTAT	360
GTGTTAAAAG ATATCTTTG TATAGTATTA CCAGATGGCC CGCCTGACCG TGTGGCCCGT	420
CCGACCGTTG ATTACGT	437

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACGTAATCAA CGGTCGAACG GGCCACACGG TCAGGCGGGC CATC

44

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATGGCCCGC CTGACCGTGT GGCCCGTCCG ACCGTTGATT ACGT

44

## (2) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2325 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ACGTAATCAA CGGTCGGACG GGCCACACGG TCAGGCAGGC CATCCCTTCG AAAACACCAAC	60
CTTGAATCAC CTACCCGAGG CTTTCAACC ACCACAAATG CCACCAAAAG CATCTATCCC	120
ATCAAAATCG CAGGTGGAGC AGGAAGGCAG GATTCTTCTT GCCATTGAAG CTATTCAGAA	180
AGGCCAAATC ACTAGTATTG GTGAAGCAGC GCGTGTATG GACGTCGCTC GAACTACTCT	240
CCAGGCTCGA TTATCTGGAC GTGTTTCGC TAAAAATATG ACCAACGCAC GTCAAAAATT	300
GTCAAATAAT GAAGAGGAAT CGCTTGTAA ATGGATCCTA TCTCTAGATA AGCGAGGAGC	360
AAGCCCCCGG CCACTTGATA TCAGAGATAT GGCTAATTG ATTATCTCTA AACGAGGTTA	420
TTCAACTGTT GAACAAGTAG GCATCAACTG GGCTTATAGC TTTGTTAAC GCCACGAATC	480
CCTACGAACT CGATTTGCTA GACGACTCAA CTATCCAAGA GCTAAAATGG AGGATCCTGA	540
AGTTATAAAA GACTGGTTCC AACGCGTACA GGAAGTTATT CAAGAGTACG GGATCTCATC	600
AGATGATATA TACAATTCG ATGAAACAGG GTTGCTATG GGAATGATTG CTACATATAA	660
AGTAGTAAC AGTTCCCAGA GGGCAGGTCG GCCGTCCCTA GTTCAACCAG GGAATCGGGA	720
ATGGGTCACT CCAATTGAGT GTATTGCTC TAATGGAGAG GTTCTACCTT CGACCCTGAT	780
CTTTAAAGGC AAAACACATC TAAAGGCATG GTATGAAGGT CAATCTATTC CTCCTACCTG	840
GAGATTTGAA GTCAGTGATA ATGGTTGGAC TACTGATAAA ATTGGACTTC GATGGCTTCC	900
AAAACACTTC ATTCCCTTGA TTAGAGGCAA ATCAGTAGGC AAATATAGCC TCCTAGTCCT	960
CGATGGCCAC GGTAGTCATT TGACACCTGA ATTGACCAA TCCTGTGCTG AAAATGAGGT	1020
TATACCTATT TGTATGCCAG CTCATTGTC CCATCTACTT CAGCCTCTTG ATGTTGGTTG	1080
TTTTAGTGTG CTTAACGCA CGTACGGAGG CATGGTTCCC AAGCAGATGC AATACGGCCG	1140
CAATCATATC GACAAGCTTG ACTTCTTAGA GGTCTATCCT AAAGCTCACC AGTGTGCTT	1200
ATCAAAGTCG AATATAATCA GTGGTTTAG AGCAACAGGT CTTGTTCTC TAGATCCTGA	1260
TCAAGTGTCTT TCTCGACTCC ATATTGCTT GAAAACACCA CCAACCCGG ATAGCCAGTC	1320
AAGTGGCTCA GTGCTTCAAA CACCACATAA TATAAAACAC CTTTGAGGAC ATCCAAAATC	1380
AGTGGAACGC CTACTTCGGA AACGGCAAGC AAGTCCAAC TCAACCTACAA ACTCTACACT	1440

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ACGTCAGCTT	CTCAAAGGGT	GTGAACTAGC	AATAACAAAC	TCAATCATAC	TGGCTAAGGA	1500
GAATGCGGAA	TTACGTGCTA	GCCATGAAAA	GCAACTACCA	AAGAGGAAGC	GTTCAAGGAA	1560
GCAGGTGATC	TATACAGAAG	GCACTACCGT	TGAAGAGGCC	CAGAGAGCTA	TACAGGAAGT	1620
GGAAGAGGTG	CAGAATGATG	AAGATATTGA	GGTTGAACCC	CAATCTCAAT	ATACGGAGAC	1680
CCCCTCGCGC	GCGCCTCCAC	GCTGCAGTAA	TTGCTTCAAT	ATAGGCCACC	GACGTACACA	1740
GTGTTCTAAA	CCACCTACTA	ATTAGTTAGA	TAGCTGTTT	TACAAGCATT	TATGTTGATT	1800
TAGAGGCCTC	ATTGGATCA	TATCGGGTAA	TCCTACCGGG	AGATGGCCCG	CCTGACCGTG	1860
TGGCCCGCCC	GACCGTTGAT	TACGTNNNNN	ACGTAATCAA	CGGTCGGACG	GGCCCCCGG	1920
TCCGGCGGGC	CATCTGGTAA	TACTATACCA	AAGATATCTT	TTTAAACATA	ATATATCTCT	1980
ACCATCCAGG	TCTAGGAGAA	TTAGATTTCT	TCTATATAGA	TTTTAAATAA	TATAAATAAT	2040
ATCTATATAC	CTTCTAAAAA	TGAATATACT	TTTACTTATG	GACTTATCAT	ATTACAATAT	2100
CTGTATTTAT	ATGTATTATA	TAAGAACATG	GTTTCATTAT	CAAAGTAAAA	ATTCTAAAAT	2160
CTGAAAAATT	CATGGAATAC	TTATTCTTAT	ATATATAACT	ATCTACAAAG	TTAGAGCTTC	2220
ATAGAAGTAG	TACTGGTTGA	TATATAATAG	AATCCCAAG	ACATCTTTA	TATGGGATTT	2280
CAGGATGGCC	GCCGACCGTG	TGGCCCGTCC	GACCGTTGAT	TACGT		2325

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 555 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met	Pro	Pro	Lys	Ala	Ser	Ile	Pro	Ser	Lys	Ser	Gln	Val	Glu	Gln	Glu
1						5			10					15	

Gly	Arg	Ile	Leu	Leu	Ala	Ile	Glu	Ala	Ile	Gln	Lys	Gly	Gln	Ile	Thr
						20			25				30		

Ser	Ile	Arg	Glu	Ala	Ala	Arg	Val	Tyr	Asp	Val	Ala	Arg	Thr	Thr	Leu
						35			40			45			

Gln	Ala	Arg	Leu	Ser	Gly	Arg	Val	Phe	Ala	Lys	Asn	Met	Thr	Asn	Ala
						50			55			60			

Arg	Gln	Lys	Leu	Ser	Asn	Asn	Glu	Glu	Ser	Leu	Val	Lys	Trp	Ile	
						65			70			75		80	

Leu	Ser	Leu	Asp	Lys	Arg	Gly	Ala	Ser	Pro	Arg	Pro	Leu	Asp	Ile	Arg
						85			90			95			

Asp	Met	Ala	Asn	Leu	Ile	Ile	Ser	Lys	Arg	Gly	Tyr	Ser	Thr	Val	Glu
						100			105			110			

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Gln Val Gly Ile Asn Trp Ala Tyr Ser Phe Val Lys Arg His Glu Ser  
 115 120 125

Leu Arg Thr Arg Phe Ala Arg Arg Leu Asn Tyr Pro Arg Ala Lys Met  
 130 135 140

Glu Asp Pro Glu Val Ile Lys Asp Trp Phe Gln Arg Val Gln Glu Val  
 145 150 155 160

Ile Gln Glu Tyr Gly Ile Ser Ser Asp Asp Ile Tyr Asn Phe Asp Glu  
 165 170 175

Thr Gly Phe Ala Met Gly Met Ile Ala Thr Tyr Lys Val Val Thr Ser  
 180 185 190

Ser Gln Arg Ala Gly Arg Pro Ser Leu Val Gln Pro Gly Asn Arg Glu  
 195 200 205

Trp Val Thr Pro Ile Glu Cys Ile Arg Ser Asn Gly Glu Val Leu Pro  
 210 215 220

Ser Thr Leu Ile Phe Lys Gly Lys Thr His Leu Lys Ala Trp Tyr Glu  
 225 230 235 240

Gly Gln Ser Ile Pro Pro Thr Trp Arg Phe Glu Val Ser Asp Asn Gly  
 245 250 255

Trp Thr Thr Asp Lys Ile Gly Leu Arg Trp Leu Pro Lys His Phe Ile  
 260 265 270

Pro Leu Ile Arg Gly Lys Ser Val Gly Lys Tyr Ser Leu Leu Val Leu  
 275 280 285

Asp Gly His Gly Ser His Leu Thr Pro Glu Phe Asp Gln Ser Cys Ala  
 290 295 300

Glu Asn Glu Val Ile Pro Ile Cys Met Pro Ala His Ser Ser His Leu  
 305 310 315 320

Leu Gln Pro Leu Asp Val Gly Cys Phe Ser Val Leu Lys Arg Thr Tyr  
 325 330 335

Gly Gly Met Val Pro Lys Gln Met Gln Tyr Gly Arg Asn His Ile Asp  
 340 345 350

Lys Leu Asp Phe Leu Glu Val Tyr Pro Lys Ala His Gln Cys Ala Leu  
 355 360 365

Ser Lys Ser Asn Ile Ile Ser Gly Phe Arg Ala Thr Gly Leu Val Pro  
 370 375 380

Leu Asp Pro Asp Gln Val Leu Ser Arg Leu His Ile Arg Leu Lys Thr  
 385 390 395 400

Pro Pro Thr Pro Asp Ser Gln Ser Ser Gly Ser Val Leu Gln Thr Pro  
 405 410 415

His Asn Ile Lys His Leu Leu Glu His Pro Lys Ser Val Glu Arg Leu  
 420 425 430

Leu Arg Lys Arg Gln Ala Ser Pro Thr Ser Pro Thr Asn Ser Thr Leu  
 435 440 445

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Arg Gln Leu Leu Lys Gly Cys Glu Leu Ala Ile Thr Asn Ser Ile Ile  
 450 455 460  
 Leu Ala Lys Glu Asn Ala Glu Leu Arg Ala Ser His Glu Lys Gln Leu  
 465 470 475 480  
 Pro Lys Arg Lys Arg Ser Arg Lys Gln Val Ile Tyr Thr Glu Gly Thr  
 485 490 495  
 Thr Val Glu Glu Ala Gln Arg Ala Ile Gln Glu Val Glu Glu Val Gln  
 500 505 510  
 Asn Asp Glu Asp Ile Glu Val Glu Pro Gln Ser Gln Tyr Thr Glu Thr  
 515 520 525  
 Pro Ser Arg Ala Pro Pro Arg Cys Ser Asn Cys Phe Asn Ile Gly His  
 530 535 540  
 Arg Arg Thr Gln Cys Ser Lys Pro Pro Thr Asn  
 545 550 555

## (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCAACCGAGT CCTCAGTATA GAC

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## (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CAACGCTTCA TAGGCGTCCA GATC

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## (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 54 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATATGAATTC ACGTAATCAA CGGTGGACG GGCCACACGG TCAGGCAGGC CATC

54

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## (2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATATGAATTTC CTTCTTGACT TCCCCGGAAC 30

## (2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ATATAAGCTT GTCACTGGAC GACATTCAG 30

## (2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2329 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ACGTAATCAA CGGTCGGGCG GGCCACACGG TCAGGGGGGC CACCCCTTCG AAAACACCAC	60
CTTGAATCAC CTACCCGAGG CTTTTCAACC ACCACAAATG CCACCAAAAG CATCTATCCC	120
ATCAAAATCG CAGGTGGAGC GGGAAAGGCAG GATTCTTCTT GCCATTGAAG CTATTGAGAA	180
AGGCCAAATC ACTAGTATTC GTGAAGCAGC GCGTGTATG GACGTCGCTC GAACTACTCT	240
CCAGGCTCGA TTATCTGGAC GTGTTTCGC TAAAAATATG ACCAACGCAC GTCAAAAATT	300
GTCAAATAAT GAAGAGGAAT CGCTTGTAA ATGGATCCTA TCTCTAGATA AGCGAGGAGC	360
AAGCCCCCGG CCACTTGATA TCAGAGATAT GGCTAATTG ATTATCTCTA AACGAGGTTA	420
TTCAACTGTT GAACAAGTAG GCATCAACTG GGCTTATAGC TTTGTTAAC GCCACGAATC	480
CCTACGAACT CGATTGCTA GACGACTCAA CTATCAAAGA GCTAAATGG AGGATCCTGA	540
AGTTATAAAA GACTGGTTCA AACGCGTACA GGAAGTTATT CAAGAGTACG GGATCTCATC	600
AGATGATATA TACAATTCG ATGAAACAGG GTTGCTATG GGAATGATTG CTACATATAA	660

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AGTAGTAACT AGTTCCCAGA GGGCAGGTCG GCCGTCCCTA GTTCAACCAG GGAATCGGGA	720
ATGGGTCACT GCAATTGAGT GTATTGCTC TAATGGAGAG GTTCTACCTT CGACCCCTGAT	780
CTTTAAAGGC AAAACACATC TAAAGGCATG GTATGAAGGT CAATCTATTC CTCCTACCTG	840
GAGATTTGAA GTCAGTGATA ATGGTTGGAC TACTGATAAA ATTGGACTTC GATGGCTTCA	900
AAAACACTTC ATTCCCTTGA TTAGAGGCAA ATCAGTAGGC AAATATAGCC TCCTAGTCCT	960
CGATGGCCAC GGTAGTCATT TGACACCTGA ATTGACCCAA TCCTGTGCTG AAAATGAGGT	1020
TATACCTATT TGTATGCCTG CTCATTCGTC CCATCTACTT CAGCCTCTTG ATGTTGGTTG	1080
TTTTAGTGTG CTTAACGCA CGTACGGAGG CATGGTTCAA AAGCAGATGC AATACGGCCG	1140
CAATCATATC GACAAGCTTG ACTTCTTAGA GGTCTATCCT AAAGCTCACCC AGTGTGCTTT	1200
ATCAAAGTCG AATATAATCA GTGGTTTTAG AGCAACAGGT CTTGTTCTC TAGATCCTGA	1260
TCAAGTGCCTT TCTCGACTCC ATATTGCTT GAAAACACCA CCAACCCCCGG ATAGCCAGTC	1320
AAGTGGCTCA GTGCTTCAAA CACCACATAA TATAAAACAC CTTTGAAAGC ATCCAAAATC	1380
AGTGGAACGC CTACTTCGGA AACGGCAAGC AAGTCCAACCT TCACCTACAA ACTCTACACT	1440
ACGTCAGCTT CTCAAAGGGT GTGAACTAGC AATAACAAAC TCAATCATAAC TGGCTAAGGA	1500
GAATGCGGAA TTACGTGCTA GCCATGAAAA GCAACTACCA AAGAGGAAGC GTTCAAGGAA	1560
GCAGGTGATC TATACAGAAG GCACTACCGT TGAAGAGGCC CAGAGAGCTA TACAGGAAGT	1620
GGAAGAGGTG CAGAATGATG AAGATATTGA GGTTGAACCC CAATCTCAAT ATACGGAGAC	1680
CCCCTCGCGC GCGCCTCCAC GCTGCAGTAA TTGCTTCAAT ATAGGCCACC GACGTACACA	1740
GTGTTCTAAA CCACCTACTA ATTAGTTAGA TAGCTGTTTT TACAAGCATT TATGTTGATT	1800
TAGAGGCCTC ATTTGATCA TATCGGGTAA TCCTACCGAG AGATGGCCCG CCTGACCGTG	1860
TGGCCCGCCC GACCGTTGAT TACGTNNNNN ACGTAATCAA CGGTCGGACG GGCCCCCGG	1920
TCCGGCGGGC CATCTGGTAA TACTATACAA AAGATATCTT TTTAACATA ATATATCTCT	1980
ACCATCCAGG TCTAGGAGAA TTAGATTCT TCTATATAGA TTTAAATAA TATAAATAAT	2040
ATCTATATAC CTTCTAAAAA TGAATATACT TTTACTTATG GACTTATCAT ATTACAATAT	2100
CTGTATTTAT ATGTATTATA TAAGAATCTG GTTTCATTAT CAAAGTAAAA ATTCTAAAAA	2160
TCTGAAAAAT TCATGGAATA CTTATTCTTA TATATATAAA CTATCTACAA AGTTAGAGCT	2220
TCATAGAAGT AGTACTGGTT GATATATAAT AGAATCAAAA AGACATCTT TATATGGGAT	2280
TTCAGGATGG CCCGCCTGAC CGTGTGGCCC GTTCGACCGT TGATTACGT	2329

## (2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 555 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Met Pro Pro Lys Ala Ser Ile Pro Ser Lys Ser Gln Val Glu Arg Glu  
1 5 10 15

Gly Arg Ile Leu Leu Ala Ile Glu Ala Ile Arg Lys Gly Gln Ile Thr  
20 25 30

Ser Ile Arg Glu Ala Ala Arg Val Tyr Asp Val Ala Arg Thr Thr Leu  
35 40 45

Gln Ala Arg Leu Ser Gly Arg Val Phe Ala Lys Asn Met Thr Asn Ala  
50 55 60

Arg Gln Lys Leu Ser Asn Asn Glu Glu Glu Ser Leu Val Lys Trp Ile  
65 70 75 80

Leu Ser Leu Asp Lys Arg Gly Ala Ser Pro Arg Pro Leu Asp Ile Arg  
85 90 95

Asp Met Ala Asn Leu Ile Ile Ser Lys Arg Gly Tyr Ser Thr Val Glu  
100 105 110

Gln Val Gly Ile Asn Trp Ala Tyr Ser Phe Val Lys Arg His Glu Ser  
115 120 125

Leu Arg Thr Arg Phe Ala Arg Arg Leu Asn Tyr Gln Arg Ala Lys Met  
130 135 140

Glu Asp Pro Glu Val Ile Lys Asp Trp Phe Lys Arg Val Gln Glu Val  
145 150 155 160

Ile Gln Glu Tyr Gly Ile Ser Ser Asp Asp Ile Tyr Asn Phe Asp Glu  
165 170 175

Thr Gly Phe Ala Met Gly Met Ile Ala Thr Tyr Lys Val Val Thr Ser  
180 185 190

Ser Gln Arg Ala Gly Arg Pro Ser Leu Val Gln Pro Gly Asn Arg Glu  
195 200 205

Trp Val Thr Ala Ile Glu Cys Ile Arg Ser Asn Gly Glu Val Leu Pro  
210 215 220

Ser Thr Leu Ile Phe Lys Gly Lys Thr His Leu Lys Ala Trp Tyr Glu  
225 230 235 240

Gly Gln Ser Ile Pro Pro Thr Trp Arg Phe Glu Val Ser Asp Asn Gly  
245 250 255

Trp Thr Thr Asp Lys Ile Gly Leu Arg Trp Leu Gln Lys His Phe Ile  
260 265 270

Pro Leu Ile Arg Gly Lys Ser Val Gly Lys Tyr Ser Leu Leu Val Leu  
275 280 285

Asp Gly His Gly Ser His Leu Thr Pro Glu Phe Asp Gln Ser Cys Ala  
290 295 300

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Glu Asn Glu Val Ile Pro Ile Cys Met Pro Ala His Ser Ser His Leu  
 305 310 315 320

Leu Gln Pro Leu Asp Val Gly Cys Phe Ser Val Leu Lys Arg Thr Tyr  
 325 330 335

Gly Gly Met Val Gln Lys Gln Met Gln Tyr Gly Arg Asn His Ile Asp  
 340 345 350

Lys Leu Asp Phe Leu Glu Val Tyr Pro Lys Ala His Gln Cys Ala Leu  
 355 360 365

Ser Lys Ser Asn Ile Ile Ser Gly Phe Arg Ala Thr Gly Leu Val Pro  
 370 375 380

Leu Asp Pro Asp Gln Val Leu Ser Arg Leu His Ile Arg Leu Lys Thr  
 385 390 395 400

Pro Pro Thr Pro Asp Ser Gln Ser Ser Gly Ser Val Leu Gln Thr Pro  
 405 410 415

His Asn Ile Lys His Leu Leu Lys His Pro Lys Ser Val Glu Arg Leu  
 420 425 430

Leu Arg Lys Arg Gln Ala Ser Pro Thr Ser Pro Thr Asn Ser Thr Leu  
 435 440 445

Arg Gln Leu Leu Lys Gly Cys Glu Leu Ala Ile Thr Asn Ser Ile Ile  
 450 455 460

Leu Ala Lys Glu Asn Ala Glu Leu Arg Ala Ser His Glu Lys Gln Leu  
 465 470 475 480

Pro Lys Arg Lys Arg Ser Arg Lys Gln Val Ile Tyr Thr Glu Gly Thr  
 485 490 495

Thr Val Glu Glu Ala Gln Arg Ala Ile Gln Glu Val Glu Glu Val Gln  
 500 505 510

Asn Asp Glu Asp Ile Glu Val Glu Pro Gln Ser Gln Tyr Thr Glu Thr  
 515 520 525

Pro Ser Arg Ala Pro Pro Arg Cys Ser Asn Cys Phe Asn Ile Gly His  
 530 535 540

Arg Arg Thr Gln Cys Ser Lys Pro Pro Thr Asn  
 545 550 555

## (2) INFORMATION FOR SEQ ID NO: 15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ACGTAATCAA CGGTCGGACG GGCCCCCGG TCAGGCAGGC CATC

44

## (2) INFORMATION FOR SEQ ID NO: 16:

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(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGATGGCCCG CCTGACCGTG TGGCCCGTTC GACCGTTGAT TACGT

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(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ACGTAATCGG TAAGCGAGTT GCCCGCGCAA GCGAGTTGCC CACC

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**What is Claimed**

1. A transposable element isolated from *Aspergillus niger* var. *awamori* comprising a DNA fragment of about 2.3 kb which comprises SEQ ID NO: 1.
2. The transposable element of Claim 1 comprising the DNA sequence of SEQ ID NO:13 or variations thereof.
3. A fragment of the transposable element of Claim 1 comprising part or all of the DNA sequence selected from the group consisting of SEQ ID NOS:1 and 16 or variations thereof.
4. An isolated transposase coded for by the transposable element of Claim 1.
5. The transposase of Claim 4 comprising the amino acid sequence of SEQ ID NO:14.
6. A method of isolating a transposable element from a filamentous fungus, comprising the steps of:
  - (a) hybridizing fungal DNA under low stringency conditions to a probe, wherein the probe comprises part or all of one of the DNA fragments of Claim 3; and
  - (b) isolating fungal DNA which hybridizes to said probe.
7. The method of Claim 6 wherein the probe comprises an imperfect direct repeat within the DNA sequence selected from the group consisting of SEQ ID NOS:1 and 16.
8. A method of isolating a transposable element from a filamentous fungus genomic library, the method comprising probing said library with an ORF-specific probe and isolating DNA which hybridizes to said ORF-specific probe.
9. A method of isolating a transposable element from a filamentous fungi, the method comprising,
  - (a) subjecting fungal DNA to polymerase chain reaction amplification using part or all of one of the DNA fragments of Claim 3 as a primer, thereby generating amplified DNA sequences;
  - (b) isolating the amplified DNA sequences; and
  - (c) optionally identifying said amplified DNA sequence.
10. A transposable element isolatable by the method of Claim 6, 7, 8 or 9.

11. A method of isolating activation sequences comprising:

- (a) inserting a marker gene within the inverted repeats of a transposable element of Claim 1 to form a modified marker gene having the structure IR-marker-IR;
- (b) inserting the modified marker gene into a DNA target;
- (c) selecting for expression of the modified marker; and
- (d) isolating DNA upstream of said modified marker gene in said DNA target, which DNA upstream of said modified marker gene comprises an activation sequence driving expression of said modified marker gene.

12. A method for inactivating a gene in a host cell wherein said gene encodes a gene product, the method comprising:

- (a) transforming a host cell with a genetic element to create a transformed host cell, wherein the genetic element comprises DNA for the gene and a transposable element of Claim 1 inserted within the DNA; and
- (b) selecting for the transformed host cells which are deficient in the gene product.

13. A method for activating a desired gene in a host cell, the method comprising:

- (a) inserting a regulatory gene within the inverted repeats of a transposable element of Claim 1 to form a modified regulatory gene having the structure IR-regulatory gene-IR;
- (b) inserting the modified regulatory gene in DNA comprising the desired gene to form a DNA construct containing the modified regulatory gene upstream of said desired gene;
- (c) transforming the host cell with the DNA construct; and
- (d) selecting for transformants having enhanced expression of said desired gene.

14. A transposable element isolated from *Aspergillus oryzae* comprising a DNA fragment of at least about 1.2 kb.

15. The transposable element of Claim 14 comprising an inverted repeat DNA sequence of SEQ ID NO:17 or a variation thereof.

16. A fragment of the transposable element of 14 comprising part of all of the DNA sequence of SEQ ID NO:17 or a variation thereof.

17. A method of isolating a transposable element from a filamentous fungus, comprising the steps of:

- (a) hybridizing fungal DNA under low stringency conditions to a probe, wherein the probe comprises part or all of one of the DNA fragments of Claim 16; and
- (b) isolating fungal DNA which hybridizes to said probe.

18. A method of isolating activation sequences comprising:

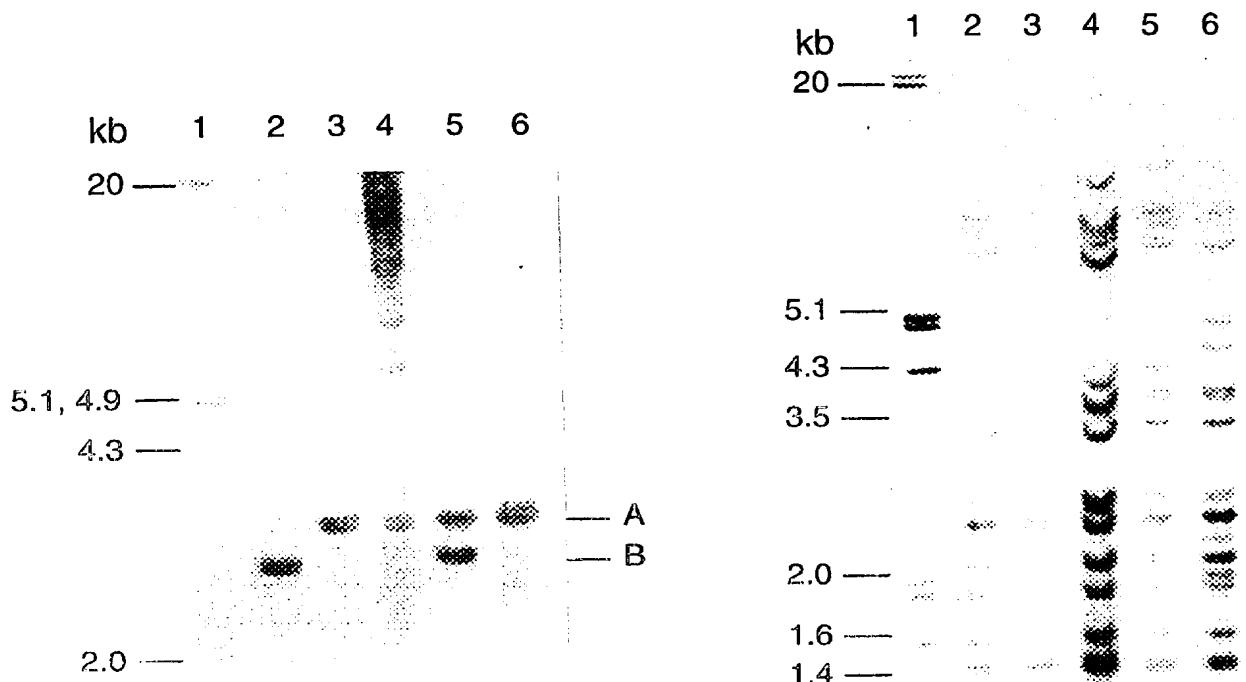
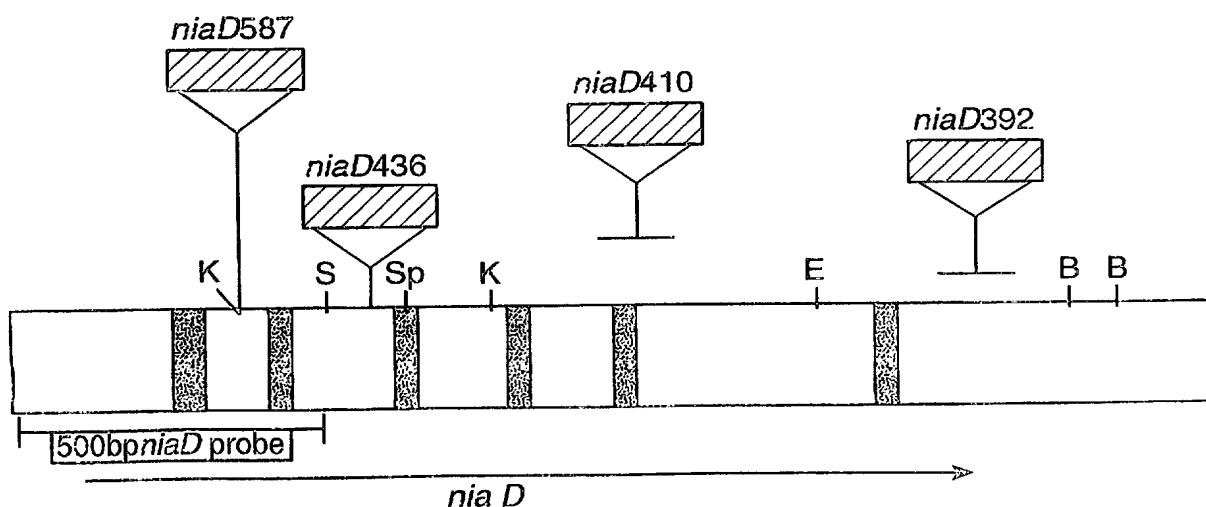
- (a) inserting a marker gene within the inverted repeats of a transposable element of Claim 10 to form a modified marker gene having the structure IR-marker-IR;
- (b) inserting the modified marker gene into a DNA target;
- (c) selecting for expression of the modified marker; and
- (d) isolating DNA upstream of said modified marker gene in said DNA target, which DNA upstream of said modified marker gene comprises an activation sequence driving expression of said modified marker gene.

19. A method for inactivating a gene in a host cell wherein said gene encodes a gene product, the method comprising:

- (a) transforming a host cell with a genetic element to create a transformed host cell, wherein the genetic element comprises DNA for the gene and a transposable element of Claim 10 inserted within the DNA; and
- (b) selecting for the transformed host cells which are deficient in the gene product.

20. A method for activating a desired gene in a host cell, the method comprising:

- (a) inserting a regulatory gene within the inverted repeats of a transposable element of Claim 10 to form a modified regulatory gene having the structure IR-regulatory gene-IR;
- (b) inserting the modified regulatory gene in DNA comprising the desired gene to form a DNA construct containing the modified regulatory gene upstream of said desired gene;
- (c) transforming the host cell with the DNA construct; and
- (d) selecting for transformants having enhanced expression of said desired gene.

**FIG. 1****FIG. 3****FIG. 2**

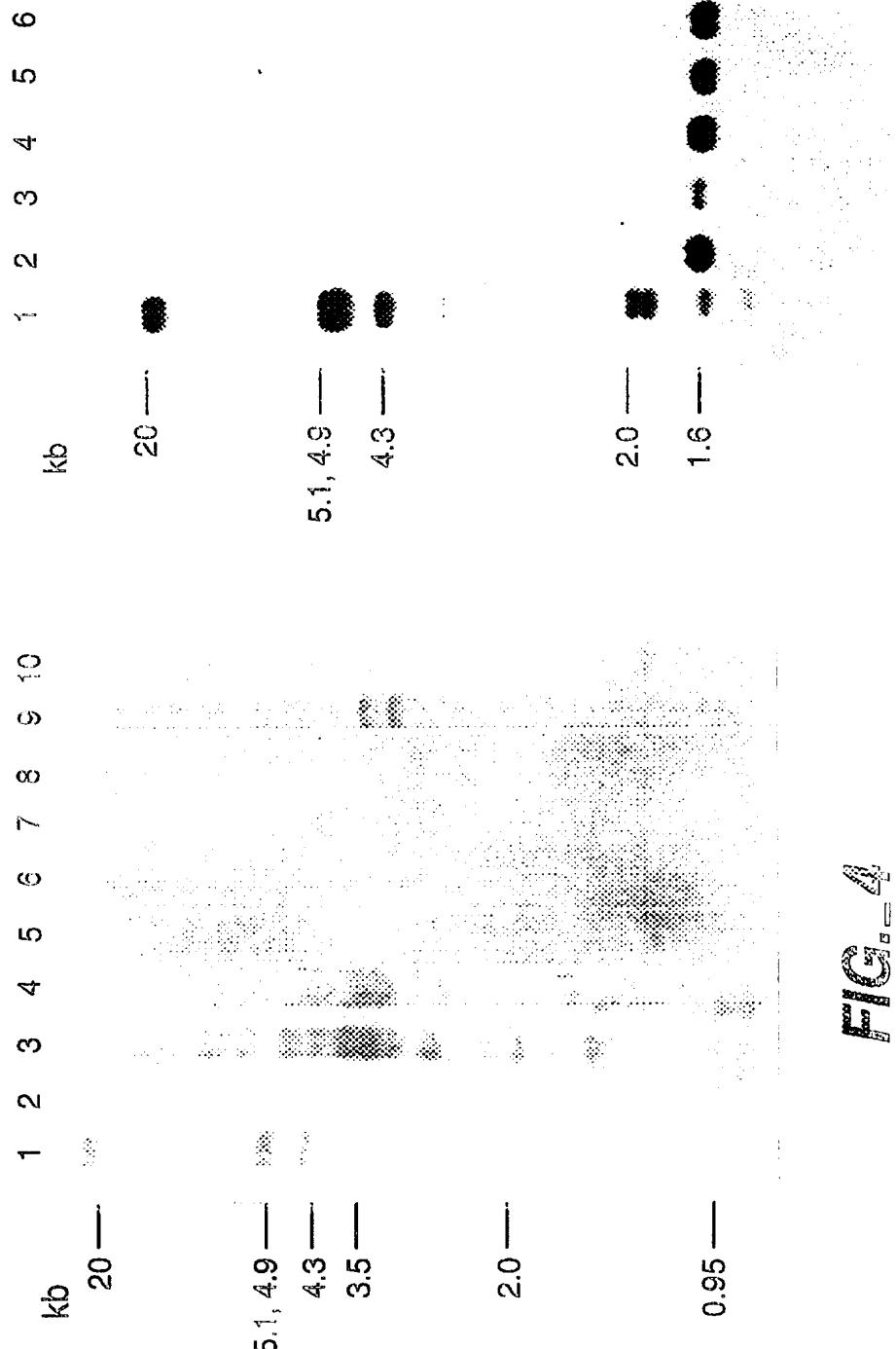


FIG. 5

FIG. 4



FIG\_6A

FIG\_6B

FIG\_6C

tgtcgacggctctggactggccaatgatggcagatatcctacggagtgcga  
agcctttgaggaaagccaagtaACGTAATCAA CGGTCGAACG GGCCACACGG  
TCAGGGCGGGC CATCCTGAAA TCCCATATAA AAGATGTCTT GGGGATTCTA  
TTATATATCA ACCAGTACTA CTTCTATGAA GCTCTAACTT TGTAGATAGT  
TATATATATA AGAATAAGTA TTCCATGAAT TTTTCAGATT TTAGAATT  
TACTTGATA ATGAAACCAG ATTCTTATAT AAAACATATA AATACAGATA  
TTGTAATATG ATAAGTCCAT AAGTAAAAGT ATATTCAATT TTAGAAGGTA  
TATAGATATT ATTTATATTA TTTAAAATCT ATATAGAAGA AATCTAATT  
TTCTAGACCT GGATGGTAGA GATATATTAT GTTTAAAAAG ATATCTTTG  
TATAGTATTA CCAGATGGCC CGCCTGACCG TGTGGCCCGT CCGACCGTTG  
ATTACGTtatgtctgcatggaggagctgataagctggtaagttaccttatcca  
tccatgcatgcagtgccctga

**FIG.\_7**

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1	ACGTAATCAA CGGTGGACG GGCCACACGG TCAGGGGGC CATCCCTCG CTTGAATCAC CTACCCGAGG CTTTTCAACC ACCACAAATG CCACCAAAG CATCTATCCC	AAAACACCAC 120
121	ATCAAATCG CAGGTGGAGC AGGAAGGCAG GATTCTCTT GCCATTGAAG CTATTCAAGA S K S Q V E Q E G R I L L A I E A I Q K	180
181	AGGCCAAATC ACTAGTATTG GTGAAGCAGC GCGTGTATT GACGTCGCTC GAACTACTCT G Q I T S I R E A A R V Y D V A R T T L	240
241	CCAGGCTCGA TTATCTGGAC GTGTGTTTCGC TAAAAATATG ACCAACGGCAC GTCAAAAT Q A R L S G R V F A K N M T N A R Q K L	300
301	GTCAAATAAT GAAGGAGGAAT CGCTTGTTAA ATGGATCCTA TCTCTAGATA AGGGAGGAGC S N N E E E S L V K W I L S L D K R G A	360
361	AAGCCCCGG CCACTTGATA TCAGAGATAT GGCTAATTG ATTATCTCTA AACGAGGTTA S P R P L D I R D M A N L I I S K R G Y	420
421	TTCAACTGTT GAACAAGTAG GCATCAACTG GGCTAATTG GCCTAATTG ATTATCTCTA AACGAGGTTA S T V E Q V G I N W A Y S F V K R H E S	480
481	CCTACGAACT CGATTGCTA GACGACTCAA CTATCCAAGA GCTAAATGG AGGATCCCTGA L R T R F A R R L N Y P R A K M E D P E	540
541	AGTTATAAAA GACTGGTTCC AACGGGTACA GGAAGTTATT CAAGAGTACG GGATCTCATC V I K D W F Q R V Q E V I Q E Y G I S S	600
601	AGATGATAAA TACAATTTCG ATGAAACAGG GTTGTCTATG GGAATGATTG CTACATAAA D D I Y N F D E T G F A M G M I A T Y K	660

**FIG.-8A**

661	AGTAGTAAC T AGT <sup>T</sup> CCAGA V V T S Q R A G R P S L V Q P G N R E	GCCAGGTCG GCCGTCCCTA GTTCAACCAG GGAATCGGGA 720
721	ATGGGTCACT CCAATTGAGT W V T P I E C I R S N G E V L P S T L I	GTATTGCTC TAATGGAGAG GTTCTACCTT CGACCCCTGAT 780
781	CTTTAAAGGC AAAACACATC F K G K T H L K A W Y E G Q S I P P T W	TAAGGCATG GTATGAAGGT CAATCTATTC CTCCCTACCTG 840
841	GAGATTGAA GTCAGTGATA R F E V S D N G W T T D K I G L R W L P	ATGGTTGGAC TACTGATAAA ATTGGACTTC GATGGCTTCC 900
901	AAAACACTTC ATTCCTTGA K H F I P L I R G K S V G K Y S L L V L	TTAGAGGCAA ATCAGTAGGC AAATATAGCC TCCTAGTCCT 960
961	CGATGCCAC GGTAGTCATT D G H G S H L T P E F D Q S C A E N E V	TGACACCTGA ATTGACCAA TCCCTGTGCTG AAAATGAGGT 6 / 13
1021	TATACCTATT TGTATGCCAG I P I C M P A H S S H L L Q P L D V G C	CTCATCTACTT CCATCTACTT CAGCCTCTTG ATGTTGGTTG 1020 13
1081	TTTAGTGTG CTTAACGCA F S V L K R T Y G G M V P K Q M Q Y G R	CGTACGGAGG CATGGTTCCC AAGCAGATGC AATACGGCG 1080
1141	CAATCATATC GACAAGCTTG N H I D K L D F L E V Y P K A H Q C A L	ACTTCTTAGA GGTCTATCCT AAAGCTCACC AGTGTGCTTT 1140
1201	ATCAAAGTCG AATATAATCA S K S N I I S G F R A T G L V P L D P D	GTGGTTTTAG AGCAACAGGT CTTGTTCCCTC TAGATCCTGA 1260

**FIG.-8B**

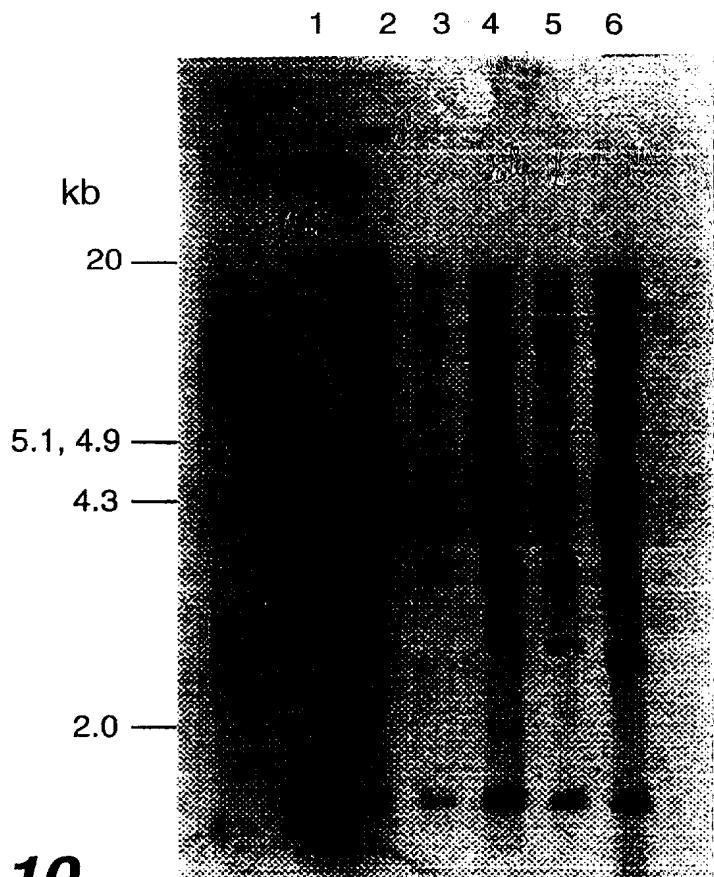
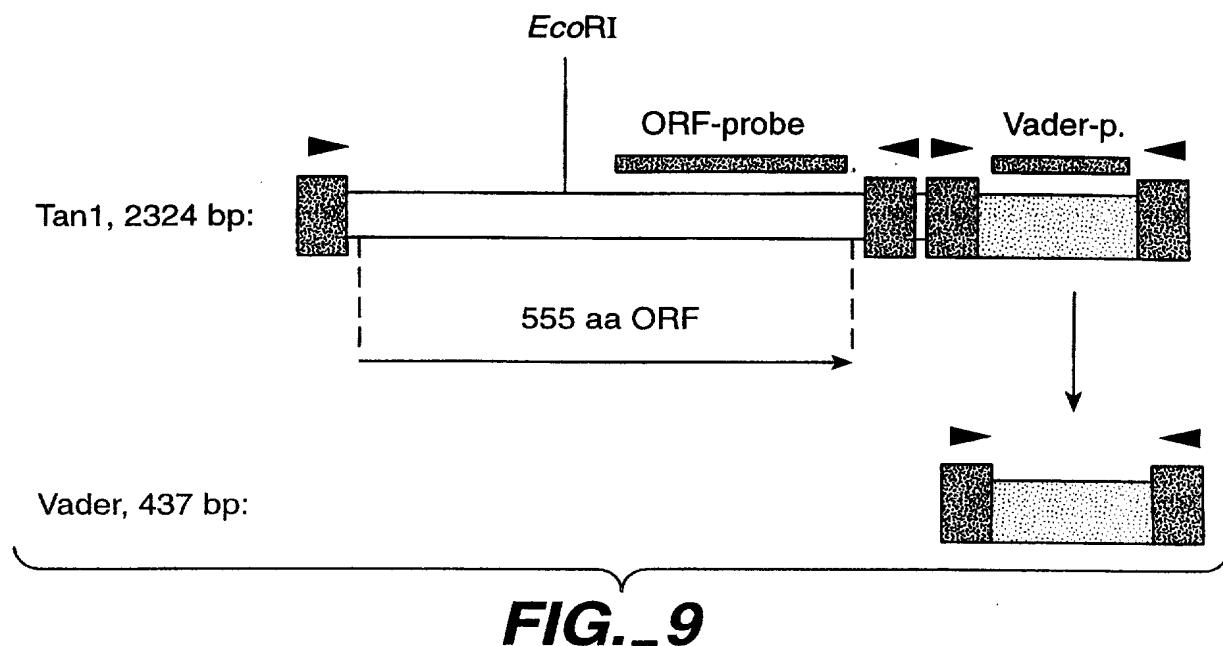
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1261	TCAAGTGCTT Q V L S R L H I R L K T P P T P D S Q S	CCAACACCCA GAAACACCCA CCAACCCCCG ATAGGCCAGTC	1320
1321	AAGTGGCTCA S G S V L Q T P H N I K H L L E H P K S	CACCACATAA TATAAACAC CTTTGGAGC ATCCAAAATC	1380
1381	AGTGGAAACGC V E R L L R K R Q A S P T S P T N S T L	AACGGCAAGC AAAGTCCAACT TCACCTACAA ACTCTACACT	1440
1441	ACGTTCAGCTT R Q L L K G C E L A I T N S I I L A K E	CTCAAAAGGGT GTGAACTAGC ATAAACAAAC TCAATCATAC	1500
1501	GAATGGGAA N A E L R A S H E K Q L P K R K R S R K	TTACGTGCTA GCCATGAAAA GCAACTACCA AAGAGGAAGC	1560
1561	GCAGGGATC Q V I Y T E G T T V E E A Q R A I Q E V	TACA GCAC TACAGGAGCTA TACAGGAAGT	1620
1621	GGAAGAGGTG E E V Q N D E D I E V E P Q S Q Y T E T	CAGAGGCC TGAAGAGGCC CAATCTCAAT ATACGGAGAC	1680
1681	CCCCTCGCGC P S R A P P R C S N C F N I G H R R T Q	GCTGCAGTAA TTGCTTCAAT ATAGGCCACC GACGTACACA	1740
1741	GTGTTCTAAA C S K P P T N *	CCACCTACTA ATTAGTTAGA TAGCTGTTTT TACAAGCATT	1800

1801	TAGAGGCCCTC	ATTTGGATCA	TATCGGGTAA	TCCTACCGGG	<u>AGATGGCCCC</u>	CCTGACCGTG	1860
1861	<u>TGGCCCGCCC</u>	GACCGTTGAT	TACGTNNNN	<u>ACGTAATCAA</u>	CGGTGGACG	GGCCCCCGG	1915
1916	<u>TCCGGGGGGC</u>	CATCTGGTAA	TACTATACCA	AAGATATCTT	TTAAACATA	ATATACTCT	1975
1976	ACCATCCAGG	TCTAGGAGAA	TTAGATTCT	TCTATATAGA	TTTTAAATAA	TATAAATAAT	2035
2036	ATCTATATAC	CTTCTAAAAA	TGAATATACT	TTTACTTATG	GACTATCAT	ATTACAATAAT	2095
2096	CTGTATTAT	ATGTATTATA	TAAGAATCTG	GTTCATTAT	CAAAGTAAA	ATTCTAAAT	2155
2156	CTGAAAATT	CATGGAATAC	TTATTCTTAT	ATATATAACT	ATCTACAAAG	TTAGAGCTTC	2215
2216	ATAGAAGTAG	TACTGGTTGA	TATATAATAG	AATCCCCAAG	ACATCTTTA	TATGGGATT	2275
2276	CAGGATGGCC	GCCGACCGTG	TGGCCCGTCC	GACCGTTGAT	TACGT		2320

**FIG.\_8D**

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**FIG.\_10**

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1 61	<u>ACGTAATCAA CGGTGGGG</u> <u>CTTGAAATCAC CTACCCGAGG</u>	<u>GGCCACACGG</u> <u>CTTTCAACC ACCACAAATG</u>	<u>TCAGGGGGC</u> <u>CACCCCTTCG</u>	<u>AAACACCCAC</u> <u>CACCCAAAG CATCTATCCC</u>	60 120
121	S K S Q V E R E G R I L A R V Y D V A R T T L	CAGGTGGAGC GGGAGGCAG GATTCTTCTT GCCATTGAAG CTATTGAGAA	180		
181	G Q I T S I R E A A R V Y D V A R T T L	ACTAGTATTG GTGAAGCAGC GCGTGTATT GACGGTCGCTC GAACTACTCT	240		
241	Q A R L S G R V F A K N M T N A R Q K L	CCAGGCTCGA TTATCTGGAC GTGTTCGCG TAAAATATG ACCAACGCAC GTCAAAATATT	300		
301	S N N E E S L V K W I L S L D K R G A	GTCAAAATAAT GAAGAGGAAT CGCTTGTTAA ATGGATCCTA TCTCTAGATA AGCGAGGAGC	360		
361	S P R P L D I R D M A N L I I S K R G Y	AAGCCCCGG CCACTTGATA TCAGAGATAT GGCTAATTG ATTATCTCTA AACGAGGTTA	420		
421	S T V E Q V G I N W A Y S F V K R H E S	TTCAACTGTT GAACAAGTAG GCATCAACTG GGCTTATAGC TTTGTTAAC GCCACGAATC	480		
481	L R T R F A R R L N Y Q R A K M E D P E	CCTACGAACT CGATTGCTA GACGACTCAA CTATCAAAGA GCTAAAATGG AGGATCCTGA	540		
541	V I K D W F K R V Q E V I Q E Y G I S S	AGTTATAAAA GACTGGTTACA AACGCGTACA GGAAGTTATT CAAGAGTACG GGATCTCATC	600		
601	D D I Y N F D E T G F A M G M I A T Y K	AGATGATATA TACATTTCG ATGAAACAGG GTTTGCTATG GGAATGATTG CTACATATAA	660		

**FIG.- 11A**

661	AGTAGTAAC T AGTCCCCAGA GGGCAGGTCG GCCGTCCCTA GTTCAACCAG GGAATCGGGA V V T S S Q R A G R P S L V Q P G N R E	720
721	ATGGGTCACT GCAATTGAGT GTATTGCTC TAATGGAGAG GTTCTACCTT CGACCCCTGAT W V T A I E C I R S N G E V L P S T L I	780
781	CTTAAAGGCC AAAACACATC TAAAGGCATG GTATGAAGGT CAATCTATTC CTCCCTACCTG F K G K T H L K A W Y E G Q S I P P T W	840
841	GAGATTGAA GTCAAGTGATA ATGGTTGGAC TACTGATAAA ATTGGACTTC GATGGCTTCA R F E V S D N G W T T D K I G L R W L Q	900
901	AAAACACTTC ATTCCCTTGA TTAGAGGCAA ATCAGTAGGC AAATATAAGCC TCCTAGTCCT K H F I P L I R G K S V G K Y S L L V L	960
961	CGATGGCAC GGTAGTCATT TGACACCTGA ATTGACCCAA TCTCGACCA S C A E N E V D G H G S H L T P E F D Q S P L D V G C	1020
1021	TATACCTATT TGTATGCCTG CTCATTGTC CCATCTACTT CAGCCTCTTG ATGTTGGTTG I P I C M P A H S S H L L Q P L D V G C	1080
1081	TTTAGTGTG CTTAACGCA CGTACGGAGG CATGGTTCAA AAGCAGATGC AATAACGGCCG F S V L K R T Y G G M V Q K Q M Q Y G R	1140
1141	CAATCATATC GACAAGCTTG ACTTCTTAGA GGTCTATCT AAAGCTCACC AGTGTGCTT N H I D K L D F L E V Y P K A H Q C A L	1200
1201	ATCAAAGTCG AATAATAATCA GTGGTTTAG AGCAACAGGT CTGTTCCCTC TAGATCCTGA S K S N I I S G F R A T G L V P L D P D	1260

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**FIG.- 11B**

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1261	TCAAGTGCTT	TCTCGACTCC	ATATTCGCTT	GAAAACACCA	CCAACCCCCG	ATAGCCAGTC		1320
	Q V L S R L H I R L K T P P T P D S Q S							
1321	AAGTGGCTCA	GTGCTTCAA	CACCACATAA	TATAAACAC	CTTTTGAAAGC	ATCCAAAATC		1380
	S G S V L Q T P H N I K H L L K H P K S							
1381	AGTGGAACGC	CTACTTCGGA	AACGGCAAGC	AAGTCCAACT	TCACCTACAA	ACTCTACACT		1440
	V E R L L R K R Q A S P T S P T N S T L							
1441	ACGTCAGCTT	CTCAAAAGGGT	GTGAACTAGC	AATAACAAAC	TCAATCATAC	TGGCTTAAGGA		1500
	R Q L L K G C E L A I T N S I I L A K E							
1501	GAATGGGAA	TTACGTGCTA	GCCATGAAA	GCAACTACCA	AAGAGGAAGC	GTTCAAGGAA		1560
	N A E L R A S H E K Q L P K R K R S R K							
1561	GCAGGGATC	TATACAGAAG	GCACTACCGT	TGAAGAGGCC	CAGAGGAGCTA	TACAGGAAGT		1620
	Q V I Y T E G T T V E E A Q R A I Q E V							
1621	GGAAAGGGTG	CAGAATGATG	AAGATATTGA	GGTTGAACCC	CAATCTCAAT	ATACGGAGAC		1680
	E E V Q N D E D I E V E P Q S Q Y T E T							
1681	CCCCTGGGC	GGGCCTCCAC	GCTGCAGTAA	TTGCTTCAAT	ATAGGCCACC	GACGTACACA		1740
	P S R A P P R C S N C F N I G H R R T Q							
1741	GTGTTCTAAA	CCACCTACTA	ATTAGTTAGA	TAGCTGTTTT	TACAAGCATT	TATGTTGATT		1800
	C S K P P T N *							

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1801	TAGAGGCCTC	ATTTGATCA	TATCGGGTAA	TCCTACCGAG	AGATGGCCCC	CCTGACCGTG
1861	TGGCCGCC	GACCGTTGAT	TACGT?????	ACGTAATCAA	CGGTTCGGACG	GGCCCCCGG
1916	TCAGGGGGC	CATCTGGTAA	TACTATACAA	AAGATATCTT	TTAAACATA	ATATACTCT
1976	ACCATCCAGG	TCTAGGAGAA	TTAGATTCT	TCTATATAGA	TTTAAATAA	TATAAATAAT
2036	ATCTATATAC	CTTCTAAAAA	TGAATATACT	TTTACTTATG	GACTTATCAT	ATTACAATAAT
2096	CTGTATTAT	ATGTATTATA	TAAGAATCTG	GTTTCATTAT	CAAAGTAAA	ATTCTAAAAA
2156	TCTGAAAAAT	TCATGGAATA	CTTATCTTA	TATATATAA	CTATCTACAA	AGTTAGAGCT
2216	TCATAGAAGT	AGTACTGGTT	GATATATAAT	AGAATCTTT	TATATGGGAT	2275
2276	TTCAGGATGG	CCCGCCTGAC	CGTGTGGCCC	GTTCCGACCGT	TGATTAACGT	2324

↓      -----      -----      -----      -----      -----

**FIG.\_11D**

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 97/14978

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 C12N15/81 C12N9/22 C12Q1/68 C12N15/11

According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>AMUTAN M ET AL: "IDENTIFICATION AND CLONING OF A MOBILE TRANSPOSON FROM ASPERGILLUS NIGER VAR. AWAMORI"          CURRENT GENETICS,          vol. 29, no. 5, 1 April 1996,          pages 468-472. XP000576438          see abstract          see page 470, right-hand column, paragraph 1 – page 471, right-hand column, paragraph 1          see page 472, left-hand column, paragraph 2          see page 473, left-hand column, paragraph 2 – last paragraph          ---          -/-</p>	1,3, 6-10, 14-16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

<sup>a</sup> Special categories of cited documents :

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- "P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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1

Date of the actual completion of the international search

5 December 1997

Date of mailing of the international search report

30/12/1997

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Montero Lopez, B

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/14978

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GLAYZER, DIANNE C. ET AL: "The isolation of Ant1, a transposable element from <i>Aspergillus niger</i> " MOL. GEN. GENET. (1995), 249(4), 432-38 CODEN: MGGEAE; ISSN: 0026-8925, 1995, XP002049285	8,10
A	see abstract see page 434, right-hand column, paragraph 2 - page 437, left-hand column, paragraph 2 ---	1-7,9, 11-20
X	MARK T. MCHALE ET AL.: "CfT-I: an LTR-retrotransposon in <i>Cladosporium fulvum</i> , a fungal pathogen of tomato" MOLECULAR AND GENERAL GENETICS, vol. 233, no. 3, June 1992, BERLIN DE, pages 337-347, XP002049286 cited in the application see abstract see page 338, left-hand column, paragraph 3 see page 338, right-hand column, last paragraph - page 339, left-hand column, paragraph 2 ---	8,10
A	TOSHITAKA MINETOKI ET AL.: "Nucleotide sequence and expression of alpha-glucosidase-encoding gene (agdA) from <i>Aspergillus oryzae</i> " BIOSCIENCE, BIOTECHNOLOGY, AND BIOCHEMISTRY, vol. 59, no. 8, August 1995, pages 1516-1521, XP002049287 cited in the application see abstract; figure 2 ---	1-5,10, 14-16
P,X	WO 96 29414 A (GENENCOR INT) 26 September 1996  see page 1, paragraph 1 see page 3, paragraph 3 - page 4, paragraph 2; figures 8-10 see page 7, paragraph 4 - page 8, paragraph 1; examples 2,4,6,7 ---	1-4, 6-10,12, 14-17,19
	-/--	

## INTERNATIONAL SEARCH REPORT

International Application No PCT/US 97/14978
---

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
P, X	<p>E. NYSSÖNEN ET AL.: "The transposable element Tan1 of <i>Aspergillus niger</i> var. <i>awamori</i>, a new member of the <i>Fot1</i> family"  <i>MOLECULAR AND GENERAL GENETICS</i>,  vol. 253, no. 1-2, 27 November 1996,  BERLIN DE,  pages 50-56, XP002049288  see abstract  see page 51, right-hand column, last  paragraph - page 53, right-hand column,  paragraph 1  see page 55, left-hand column, paragraph 3  - right-hand column, paragraph 2  -----</p>	1-10, 14-16

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

International Application No

PCT/US 97/14978

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9629414 A	26-09-96	NONE	